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Transcriptional networks of the stress responses in the human pathogen *Candida glabrata*

Antonin Thiébaud

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Sorbonne Université

Institut de Biologie Paris-Seine - Team Structure of Genes Networks

Doctoral school : Complexité du Vivant

Doctoral Thesis

**Transcriptional networks of the stress responses in
the human pathogen *Candida glabrata***

Publicly defended on December 5th, 2018

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For Andrée, Jean, Léon and Robert.

“I would rather have questions that can't be answered than answers that can't be questioned.”

Richard Feynman

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Abstract

Candida glabrata is simultaneously a commensal of human gut and a pathogenic yeast with an increasing prevalence. It is often associated with fatal bloodstream infections, notably because of its ability to resist azole treatments, evade the immune system and easily colonize the human host. Also, it displays incredible abilities to adapt and resist adverse growth conditions. However, little is known about *C. glabrata* capacities to adapt and the underlying regulations. This assessment led to the implementation of the Candihub project. It aims to describe the mechanisms that allow *C. glabrata* to survive and thrive as a pathogen and has a focus on the transcriptional regulatory networks promoting the yeast strong resistance to stress.

My PhD project was undertaken within the framework of Candihub. I tried to unravel the regulation networks associated to several transcription factors. These factors were chosen because of their key roles in controlling an array of stress responses : iron deprivation, iron excess, oxidative stress, osmotic stress... To achieve that goal, I performed high-throughput transcriptomic (microarrays) and genomic (ChIP -seq) analyses. This led to the construction of a wide network of interactions. Afterwards, I focused on smaller parts of this network.

The first part tackled the role of the CCAAT-Binding Complex in respiration and iron homeostasis. The CBC is very conserved across the fungi. In *S. cerevisiae*, it controls cellular respiration, while in pathogenic fungi such as *C. albicans*, it controls the iron homeostasis. We showed that the CBC has a dual role in *C. glabrata* : it interacts with the regulatory subunit Hap4 to control respiration and it collaborates with Yap5 to act on iron homeostasis.

The second part was based on the use of comparative transcriptomics to uncover unknown features of the iron starvation response of *C. glabrata*. We demonstrated the significance of Aft2 in response to iron starvation and we identified the regulatory network of Aft2. This revealed the involvement of genes responsible for ribosome rescue in the No GO Decay pathway, thus suggesting a link between iron homeostasis and the NGD.

Résumé

Candida glabrata est à la fois un commensal de l'homme et une levure pathogène dont la prévalence est en train d'exploser. Elle est souvent cause d'infections systémiques mortelles, notamment en raison de ses aptitudes à résister aux azoles, limiter sa détection par le système immunitaire et facilement coloniser l'hôte humain. Par ailleurs, elle possède une incroyable capacité à s'adapter et résister aux conditions de croissance défavorables. Cependant, on ne sait que très peu de choses sur les capacités d'adaptation de *C. glabrata* et les régulations qui les sous-tendent. Ce constat a mené à la création du projet Candihub, qui a pour but de décrire les mécanismes permettant à ce champignon de survivre et prospérer en tant que pathogène de l'homme. Candihub se focalise sur les réseaux de régulations transcriptionnelles favorisant la forte résistance au stress de *Candida glabrata*.

Mon projet de thèse s'est déroulé dans le cadre de Candihub. J'ai révélé les réseaux de régulation associés à plusieurs facteurs de transcription. Ces facteurs ont été spécialement choisis en raison de leurs rôles clés dans le contrôle de diverses réponses au stress, telles que la carence en fer, l'excès de fer, les stress oxydatif et osmotique... Dans ce but, j'ai réalisé des expériences haut-débit de transcriptomique (puces à ADN) et génomique (ChIP-seq). Cela a mené à la construction d'un vaste réseau d'interactions. Je me suis ensuite concentré sur des sous-ensembles de ce réseau.

La première partie a abordé le rôle du CCAAT-Binding Complex dans la respiration et l'homéostasie du fer. Le CBC est très conservé parmi les champignons. Dans *S. cerevisiae*, il contrôle la respiration cellulaire tandis que dans les champignons pathogènes tels que *C. albicans*, il gère l'homéostasie du fer. Nous avons montré que le CBC a un double rôle dans *C. glabrata* : il interagit avec la sous-unité régulatrice Hap4 pour contrôler la respiration et il collabore avec Yap5 pour réguler l'homéostasie du fer.

La deuxième partie est fondée sur l'utilisation de la transcriptomique comparative pour découvrir de nouvelles propriétés de la réponse à la carence en fer de *C. glabrata*. Nous avons démontré l'importance d'Aft2 dans la réponse à la carence en fer et identifié le réseau de régulation d'Aft2. Cela a révélé le rôle de gènes normalement impliqués dans le sauvetage des ribosomes dans la voie du No Go Decay, ce qui suggère l'existence d'un lien entre l'homéostasie du fer et le NGD.

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List of Abbreviations

aa	amino-acid
BPS	Batho Phenanthroline diSulfonate
CBC	CCAAT Binding Complex
CGD	CCandida Genome Database
ChIP	Chromatin Immuno-Precipitation
ESR	Environmental Stress Response
GO	Gene Ontology
GRN	Gene Regulatory Networks
HAP	Heme Activator Protein
OSR	Oxidative Stress Response
SGD	Saccharomyces Genome Database
TF	Transcription Factor
WGD	Whole Genome Duplication
WT	Wild Type
YAP	Yeast Activator Protein
YPD	Yeast extract Peptone Dextrose

Introduction

Candida glabrata, a yeast with many faces

Candida glabrata is an interesting model organism. Despite its name, it is phylogenetically closer to *Saccharomyces cerevisiae* than to other *Candida* species. It gained interest a few years ago when its pathogenic potential was fully revealed. But even now, a lot is still unknown about this yeast. For example, we still don't know whether it is asexual and if it really is commensal of the human host. All these questions will be tackled in the following part.

1.1 A hectic phylogenetic classification

The classification of the haploid hemiascomycete *Candida glabrata* has been quite vibrant for a long time. Due to the resemblance with some previously discovered fungi, it was first called *Cryptococcus glabratus* after its discovery on grapes (Berlese, 1894) and in human stools (Anderson, 1917), a century ago. Its name switched to *Torulopsis glabrata* in 1938 (Lodder et al., 1938), after the authors noticed that the yeast wasn't able to form pseudomycelium and had morphological and physiological characteristics resembling the ones of *Torulopsis* yeasts. Then, it changed to *Candida glabrata* in 1978 (Yarrow et al., 1978), following an important change in yeast classification and the disappearance of the *Torulopsis* genus. Finally, it was attributed to the *Nakaseomyces* clade by Kurtzman et al., 2003, after they performed a huge phylogenetic work thanks to the study of several genes, including rDNA genes, translation elongation factor, actin, RNA polymerase and mitochondrial genes. This clade also comprises three environmental species (*N. bacillisporus*, *N. delphensis*, *C. castelli*) added by Kurtzman et al., 2003, and two other pathogenic species (*C. nivariensis* and *C. bracarensis*) were added later to that clade (Alcoba-Flórez et al., 2005; Correia et al., 2006).

1.2 Close to *Saccharomyces spp*, far from *Candida spp* ?

The phylogenetic study of Kurtzman et al., 2003 was soon followed by the publication of the whole genomic sequence of *C. glabrata* ATCC2001 strain by the Genolevures consortium (Dujon et al., 2004). They revealed that *C. glabrata* genome is composed of 13 chromosomes, totaling around 12.3 Mb. The genome has an average GC content of 38.8% and encodes for 5283 CDS. It resembles the overall structure of *S. cerevisiae* which is 12.1 Mb long, has an average GC content of 38.3% and encodes for 5807 CDS. Some other genomic similarities between the two species can be found in Table 1.1. This article highlighted the closeness of *C. glabrata* and *S. cerevisiae*. They both underwent a Whole Genome Duplication (WGD) event (Wolfe et al., 1997; Dietrich et al., 2004; Kellis et al., 2004) before their divergence as distinct species, as shown by the shared duplicated blocks of sister chromosomal regions (Lalo et al., 1993; Dujon et al., 2004). *C. glabrata* also displays exactly the same set of 42 tRNA encoding genes than *S. cerevisiae*. The two species even share an average of 65% of sequence identity between orthologous proteins ; this is translated by the fact that approximately 4800/5300 genes in *C. glabrata* have an homologue in *S. cerevisiae* (Gabaldón et al., 2016).

Species	<i>S. cerevisiae</i>	<i>C. glabrata</i>
Genome size (Mb)	12.1	12.3
Average GC content (%)	38.3	38.8
Total CDS	5,807	5,283
Total tRNA genes	274	207
Average gene density (%)	70.3	65.0
Average GC in CDS (%)	39.6	41.0
Average CDS size (codons)	485	493
Median CDS size (codons)	398	409
Maximum CDS size (codons)	4,911	4,881

TABLE 1.1. General characteristics of *S. cerevisiae* and *C. glabrata* genomes. This table is adapted from Dujon et al., 2004.

However, as close as they may be, this 35% discrepancy between these two yeasts is the same than the difference between human sequences and zebrafish (Gabaldón et al., 2013). In a general way, *Nakaseomyces spp* genomes are smaller and contain fewer genes than *S. cerevisiae* (Gabaldón et al., 2013). One explanation could be that *C. glabrata* has a much higher rate of loss of duplicated genes (Dujon et al., 2004). According to this team, *C. glabrata* lost so much paralogues that it resulted in reductive evolution, associated with loss of functions and decrease genome redundancy. Even more striking, *C. glabrata* genome redundancy is equivalent to that of *Kluyveromyces lactis*, a pre-WGD species. Mainly, the genes were lost in galactose metabolism, phosphate metabolism, cell rescue, defence and virulence

and nitrogen and sulphur metabolism compared to *S. cerevisiae* and three other yeast species. Among other genomic differences, *S. cerevisiae* genome contains active transposon (Krastanova et al., 2005), while all *Nakaseomyces* species, including *C. glabrata* have transposon-free genomes (Gabaldón et al., 2013). Last but not least, *C. glabrata* is pathogenic while *S. cerevisiae* isn't, which indicates some profound genomic differences in these yeast. As mentioned previously, *C. glabrata* has some specific genes with no homology to *S. cerevisiae*. Though one could have thought that these specific genes might be an explanation as to why *C. glabrata* is pathogenic, while *S. cerevisiae* is not, Gabaldón et al., 2016 indicated that most differences in gene content was not related to virulence.

Despite these differences and the fact that *Candida albicans* is also pathogenic, *C. glabrata* is even more distant from this yeast than it is from *S. cerevisiae* (Dujon et al., 2004; Fitzpatrick et al., 2006), as represented in Figure 1.1. Another major difference between *C. glabrata* and *C. albicans* is the change in *C. albicans* genetic code : in this species, the codon CTG is translated as a serine, while in other yeasts, including *S. cerevisiae* and *C. glabrata*, it is translated as a leucine.

C. albicans and *C. glabrata* have a common ecological niche, they can both cause infections in human ranging from benign to fatal and they even share a common species name. However, despite these common points, they still are very distant species according to their genomic sequences as well as some of their phenotypic features. For example, *C. glabrata* cells are usually described as budding yeast or pseudohyphae (under certain specific conditions such as limiting nitrogen conditions (Csank et al., 2000)), while *C. albicans* cells can be shaped into yeast, pseudohyphae, hyphae or chlamydo spores. A more complete comparison of several features in these two yeasts and *S. cerevisiae* can be found in Table 1.2. Nevertheless, these two *Candida* species are both pathogen, and this pathogenicity had a big impact in bringing them under the focus of research.

1.3 An "emerging" opportunistic pathogen

Candida glabrata began to draw attention only long after its discovery, even though it was identified as early as the beginning of the 20th century. It only received significant consideration in the late 80's, when it was noticed to be one of the main cause of fungal infections in immunocompromised individuals (Just et al., 1989). Following this statement, the increasing incidence of this yeast was acknowledged in the late 20th century when it was declared as an emerging pathogen (Hazen, 1995). It was also noticed

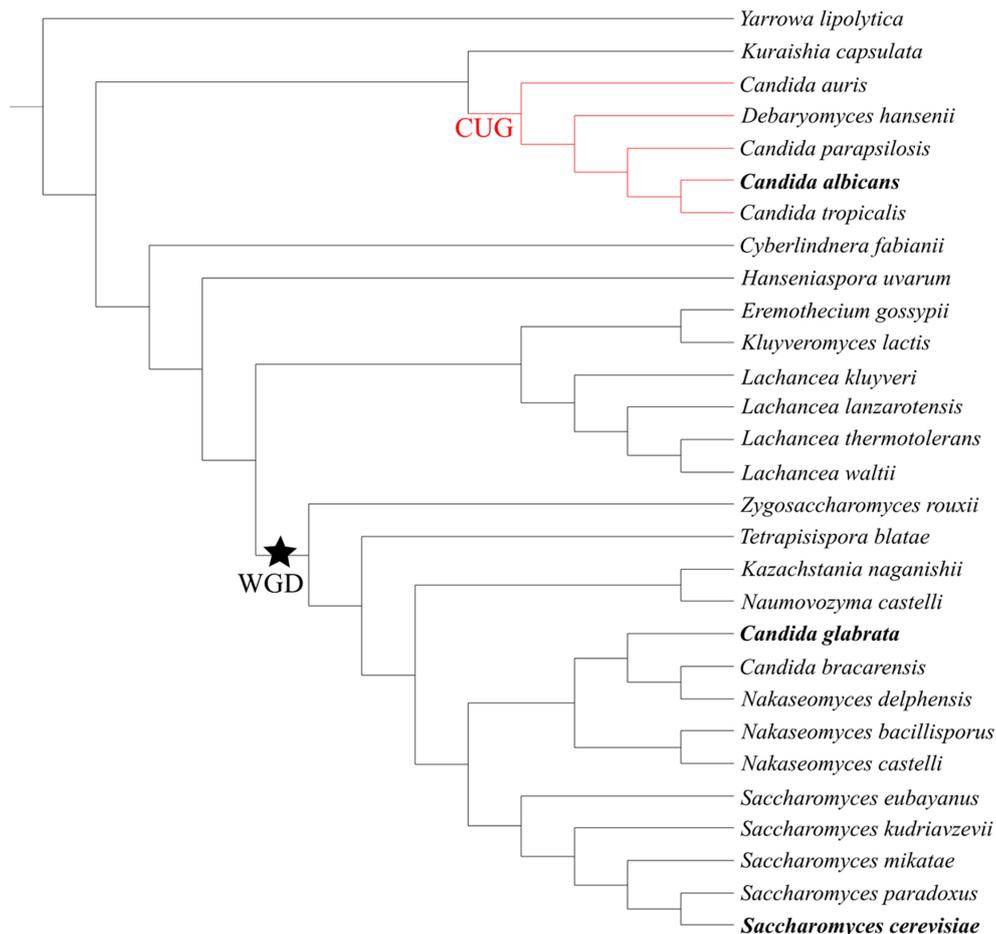


FIGURE 1.1. Phylogenetic tree of the *Saccharomycotina* clade. Schematic representation based of the phylogenetic tree inferred by X.-X. Shen et al., 2016. The tree was inferred from the concatenation-based analysis of 1233 single-copy orthologues. The Whole Genome Duplication is indicated by a black star. The CUG-clade is depicted by red branches on the tree. *C. glabrata*, *C. albicans* and *S. cerevisiae* are highlighted in bold.

that its prevalence tends to increase with age, antibiotic treatment, length of stays in hospitals, diabetes mellitus. . . (Angoulvant et al., 2016).

Usually, *Candida glabrata* is a harmless fungi of the human gut microbiota. However, given some specific conditions, it can proliferate and trigger superficial benign infections such as oral or vaginal thrush, gastrointestinal tract infection or urinary bladder infection (Pfaller et al., 1998; Fidel et al., 1999), hence the adjective “opportunistic”. In the worst cases, it can breach the mucosal barriers, enter the bloodstream and disseminate throughout the body, causing systemic candidiasis infections with high mortality rates (40-60%), especially in immunocompromised individuals (cancer patients undergoing chemotherapy, transplanted patients, for example) and elder people (Angoulvant et al., 2016).

Systemic candidiasis are now reported to account for 75% of all invasive fungal infections (D.L. Horn

Feature/Species	<i>C. glabrata</i>	<i>C. albicans</i>	<i>S. cerevisiae</i>
Ploidy	Haploid	Diploid	Diploid
Virulence	Opportunistic pathogen	Opportunistic pathogen	Non-pathogenic
Major sites of infection	Oral, vaginal, disseminated	Oral, vaginal, disseminated	Non-infectious
Mating genes	Present	Present	Present
Sexual cycle	Unknown	Known (cryptic)	Known
Clonal population structure	Yes	Yes	No
Phenotypic switching	Present	Present	Absent
True hyphae	Absent	Present	Absent
Pseudohyphae	Present	Present	Present
Biofilm formation	Present	Present	Present
Major adhesins	Lectins (<i>EPA</i> genes)	Lectins Hwp1 adhesin Als adhesins	Lectins (<i>FLO</i> genes) Sexual agglutinins
Auxotrophy	Niacin, thiamine, pyridoxine	None	None
Azole resistance	Innate resistance	Susceptible	Susceptible
Mitochondrial function	Petite positive	Petite negative	Petite positive

TABLE 1.2. Comparison of *C. glabrata*, *C. albicans* and *S. cerevisiae*. ECM : extracellular matrix ; Hwp1 : hyphal cell wall protein. This table is adapted from Kaur et al., 2005.

et al., 2007; Azie et al., 2012) and *Candida spp* are in the top five causes of nosocomial bloodstream infections (Gudlaugsson et al., 2003; Pfaller et al., 2007). Most of the time, *Candida albicans* is the main cause of candidiasis (50-70%), followed by *C. glabrata* (20-30%) (Perlroth et al., 2007). However, the role of *C. glabrata* as cause of candidiasis is rapidly increasing, perhaps because of its natural resistance to the compounds usually administered as treatment of this disease, namely azoles (Jandric et al., 2011; Pfaller, 2012). It can also easily acquire resistance to another class of antifungal, the echinocandins. It was shown that resistance increases in case of a pre-treatment. Additionally, a phenomenon of cross-resistance to other drugs can appear in strains already resistant to fluconazole (Komshian et al., 1989; Malani et al., 2005). One of the principal cause of the innate resistance of *C. glabrata* is the increased efflux of drugs driven out of the cells by pumps, usually regulated by *PDR1*. When this gene is over-expressed, the cells tend to bear an increased resistance and a increased virulence. Also, some changes in the cell wall have been reported to prevent drug diffusion (Parkinson et al., 1995; Clark et al., 1996; Vermitsky et al., 2004; Ferrari et al., 2011a). All these mechanisms are reviewed in Sanglard, 2002.

C. glabrata and *C. albicans* can both easily infect humans, however their strategies are different on several points (reviewed in Brunke et al., 2013) :

Adhesion : *C. albicans* have Als and Hwp adhesins, while *C. glabrata* has Epa adhesins. Also, both species can form biofilms on host cells or medical devices (Iraqi et al., 2005; Nobile et al., 2006).

Invasion : *C. albicans* mainly use its hyphae form to gain access to epithelial cells. The invasion mechanism in *C. glabrata* is not known yet, as this yeast doesn't form true hyphae. However,

it was noticed that *C. glabrata* infections cause reduced inflammatory reaction from the host, contrary to *C. albicans*. It might suggest a smoother way for *C. glabrata* to enter its host.

Interaction with immune cells : The two species have very different approach : shortly after internalization in macrophages, *C. albicans* uses hyphae to burst them, while *C. glabrata* uses the phagosome as a safe haven to multiply before bursting it. Once again, this has the advantage to be more quiet against the immune system of the host.

Nutrient acquisition : *C. albicans* is much better equipped to face the host in nutrients acquisition. It has no known auxotrophy and owns several integration pathways for a lot of nutrients, including metallic ions. On the other hand, *C. glabrata* loss of genes after WGD caused the disappearance of several important pathways (mentioned in Section 1.2). This lack has to be compensated for the cells to survive and thrive.

All these mechanisms are summarised in Figure 1.2. Finally, given these dissimilarities and the phylogenetic disparity between *C. glabrata* and *C. albicans*, it is likely that their pathogenicity and their infection strategies came from different and independent evolutions.

1.4 *C. glabrata* genome plasticity : a huge impact

Over the years, it became clear that *Candida glabrata* possesses an impressive genome plasticity and can be rearranged easily. Several teams reported some interesting features and consequences to this very dynamic nature of the *C. glabrata* genome, which could go as far as considering the rearrangements as an adaptive mechanism. The first clue might be the high rate of loss of paralogues after the WGD, leading to a reductive evolution (Dujon et al., 2004) (Table 1.1). Shin et al., 2007 reported that changes in karyotype can appear quickly in strains isolated at different time intervals in the same patient without antifungal therapy. They also showed that these strains were able to acquire azole resistance under treatment with azole compounds. Ahmad et al., 2013 reported that the number of chromosomes of *C. glabrata* can vary among different isolates. Especially, they found extra chromosomes created by segmental duplication and translocation. They supposed that it was a way for *C. glabrata* to improve its fitness around new environments. Poláková et al., 2009 also noticed some size variation in the chromosomes, but more importantly, they remarked that new supernumerary chromosomes carried duplicated genes among which

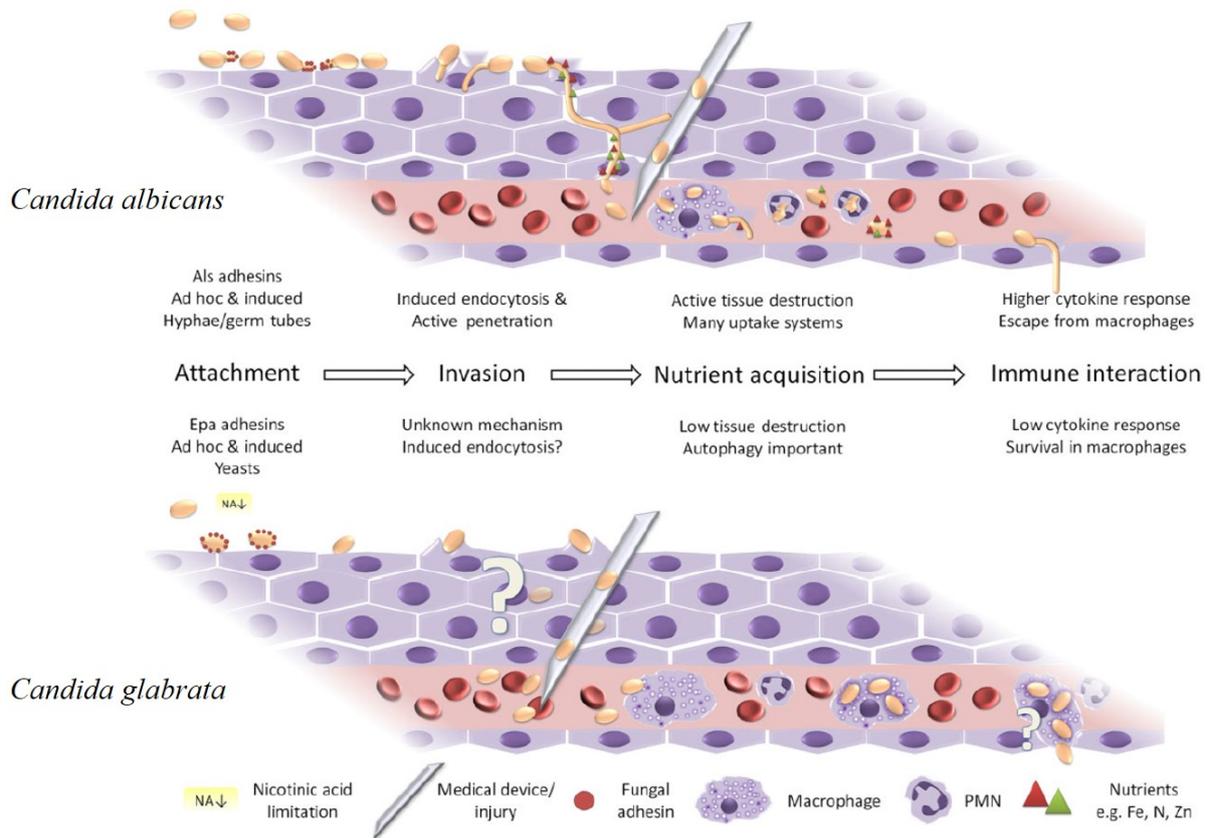


FIGURE 1.2. Infection strategies of *C. glabrata* and *C. albicans*. These two yeasts have very different methods to infect the host. However, there is still a lot of unknown processes involved in *C. glabrata* pathogenicity. Adapted from Brunke et al., 2013.

the orthologues of *S. cerevisiae* family of ABC transporters, which plays a role in pleiotropic drug resistance and yeast-host interaction genes. Overall, these karyotype variations correlated with antifungal drug resistance.

C. glabrata genome contains the family of EPA genes (homologues of the *FLO* genes in *S. cerevisiae*), coding for cell-wall proteins, which are known to be responsible for the adherence to human epithelium (Cormack et al., 1999; Gabaldón et al., 2013). Coincidentally, Muller et al., 2009 reported size variation in tandem arrays of repeated genes, which often encode the EPA genes, suggesting a role in adaptation to the host (Figure 1.3). It also suggests a role in virulence because these tandem repeats were also found to encode aspartyl proteases responsible for adherence to mammalian cells and survival in macrophages (Kaur et al., 2007).

However, this genomic changes also happen very quickly in lab strains : Bader et al., 2012 noticed some karyotypic modifications that could be associated to phenotypic variations. It shows that genome

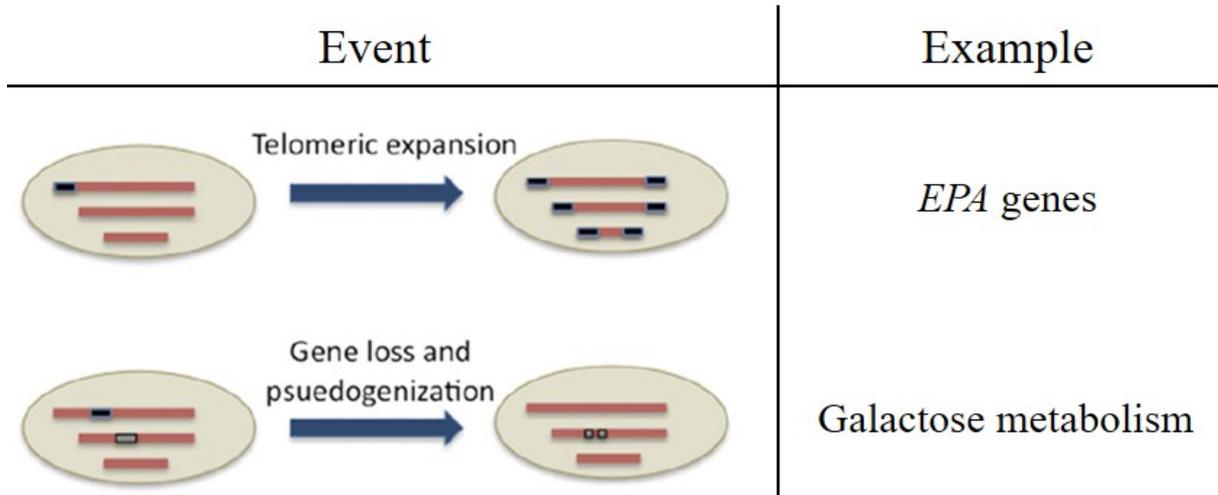


FIGURE 1.3. Genomic processes responsible for the evolution of *C. glabrata*. *EPA* genes : family of GPI-anchored cell wall proteins that facilitate host recognition and *C. glabrata* adherence. Adapted from Moran et al., 2011).

plasticity occurs not only during the harsh conditions of host infection, but also in laboratory conditions during a very short time and without strong selective pressures.

Hence, it seems that chromosome remodeling and gene duplications play an important role in the specialization to specific environments, virulence, and interaction with the host (Butler et al., 2009; Moran et al., 2011; Gabaldón et al., 2018).

1.5 Breaking *C. glabrata* clichés

1.5.1 A commensal yeast ?

Candida glabrata is often considered as a human commensal, even if its prevalence can vary a lot between studies and is influenced by a range of factors, including the age, the previous use of antifungal treatments and the medical condition of a patient. *C. glabrata* has been detected in human flora (oropharyngeal, digestive, vaginal), on medical devices (Iraqi et al., 2005), phones (Kordecka et al., 2016) but it can also be found in non-human related niches such as fermenting coffee beans (de Melo Pereira et al., 2014) or cloaca of several bird species (Cafarchia et al., 2008; Francesca et al., 2014; Al-Yasiri et al., 2016). These are all diverse isolation sites, sometimes without any link between them (for example, mobile phone and bird cloaca or with human (for example, bird cloaca and human). It suggests that either *C. glabrata* is a commensal of several sources, or that it contaminated all these secondary sources from a primary source that is still unknown. This supposition is coherent with the fact that *C. glabrata*

is not always found in these niches. This type of behaviour has been shown for other yeasts, such as *C. albicans* (Bensasson et al., 2018) or *S. cerevisiae* (Goddard et al., 2015).

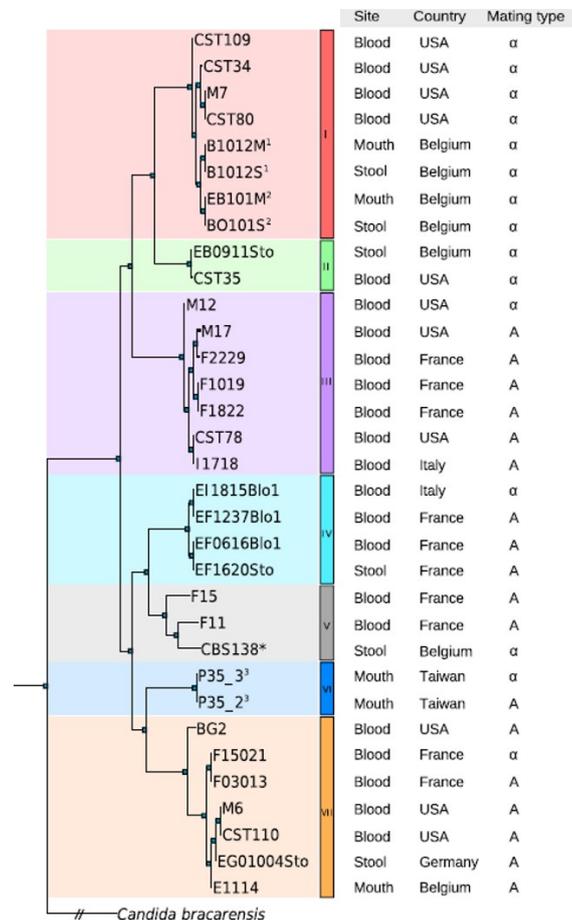


FIGURE 1.4. Phylogenetic structure of 33 Strains of *C. glabrata*. This tree indicates the phylogenetic structure of the strains based on SNPs data analysis. Total number of SNPs isn't displayed, but a range of 4.66 to 6.56 SNPs per kb per strain when compared to the reference strain (as sequenced by Dujon et al., 2004) is observed. Each colour designates a clade. Super-indices indicate pairs of strains isolated in the same patient, but on different body site or at a different date. Body site and country of the isolation are displayed, along with the mating type of the strains. Adapted from Carreté et al., 2018.

One strong argument in favour of the human commensality of *C. glabrata* is the existence of phylogenetically distinct clades among the yeast population related to specific geographical origins. In other words, some distinct clades were defined according to genetic and phenotypic markers, and these clades were specific to a geographical zone (Dodgson et al., 2003; Dodgson et al., 2005; Brisse et al., 2009; Rolland et al., 2010; Schwarzmüller et al., 2014). However, these studies were only performed on a few markers, and consequently, the number of detected clades varied a lot. This problem was solved by the study of Carreté et al., 2018, who assessed genomic and phenotypic variations in 33 *C. glabrata* isolates coming from all over the world. They were able to sequence these strains and class them into tree with seven clades (Figure 1.4). On this tree, it appears quite clear that strains of the same clade clustered

together despite their geographical origin (for example, cluster I with USA and Belgium), which would go against a human commensality of *C. glabrata* : this undermines the idea of a genomic co-evolution between *C. glabrata* and human, as we could have expected if there was a commensal relationship between them. It lets us think that human activities and transportations caused the crossing of strains that have been isolated for a long time, most likely in specific geographical areas.

However, *C. glabrata* displays several traits that let us think of it as a commensal of human. The optimal growth temperature of *C. glabrata* is close to 37°C, the normal internal temperature of the human body. This is a huge advantage as both a commensal and an opportunistic pathogen. *C. glabrata* has a higher stress resistance and an enhanced ability to sustain prolonged starvation. It can withstand very high concentrations of H₂O₂ (also considered as oxidative stress), varying concentration of bio-available iron, lack of oxygen, among others, and can also resist to shortage of nutrients caused by protective mechanisms of the human host. Especially, the adaptation to use alternative nutrients and survive long periods of starvation is essential for *C. glabrata* to survive macrophage engulfment (Roetzer et al., 2010) and use the macrophage as a “Trojan horse” to invade the whole body of the host. This type of adaptation reminds the bacteria surviving internalization in amoebae and the subsequent oxidative and osmotic stresses (Greub et al., 2004), as well as the likely selection for bacterial virulence and resistance traits (Tosetti et al., 2014; Hao et al., 2015). Additionally, earlier, *C. glabrata* can also resist to antifungal drugs and acquire new resistances, which is an useful mechanisms for a human pathogen. Also, *C. glabrata* genome has remodeled its cell-wall components resulting in a higher adherence. The ability to adhere to the host tissues is mediated by cell-wall-associated proteins called adhesins (reviewed in Groot et al., 2013). This translates into the presence of a high number of particular adhesins encoded by the *EPA* family of genes . This adhesins proved to be crucial in virulence and to form biofilms (Cormack et al., 1999; Roetzer et al., 2011a), an ability which increases resistance, virulence and persistence in the host. Finally, *C. glabrata* has lost several hundreds of genes, including genes in the pyridoxine, thiamine and nicotinic acid biosynthetic pathways (Dujon et al., 2004; Kaur et al., 2005). The fact that the human host is a really stable growth environment, because all its parameters (temperature, oxygen, nutrients...) are regulated through homeostasis, could be an explanation to the loss of specific pathways in *C. glabrata*. It also means that this yeast has a higher dependence of the host, and rely on it to acquire nutriments. All these features tend to show an adaptation of *C. glabrata* to its human host.

Nonetheless, several of these features (growth at 37°C, loss of the nicotinic acid pathway and presence of auxotrophies) are shared by all *Nakaseomyces* species, pathogenic and non-pathogenic, and the

expansion of the *EPA* family was linked to virulence in the pathogenic *Nakaseomyces* (*C. glabrata*, *C. bracarensis*, *C. nivariensis*). This means that not all these changes are specific to *C. glabrata* and its commensalism (or its pathogenicity). Hence, *C. glabrata* clearly displays commensal traits but the repercussions of this commensalism on *C. glabrata* genome are not clearly identified yet. This could be because *C. glabrata* might be a recent human commensal (which hasn't left genomic traces yet) or/and because we still lack experimental data on the comparisons between *C. glabrata* and other *Nakaseomyces*.

1.5.2 An asexual yeast... for now

C. glabrata is believed to be an asexual haploid fungus, which reproduces exclusively by budding. Though it is haploid and can bud, it might not be as asexual as we thought, even if its mating has never been actually observed. Yeast in the same clade than *C. glabrata* and *S. cerevisiae* often have two mating types in haploid cells. These haploid cells can merge, forming diploids, which can then undergo meiosis and sporulation (Muller et al., 2008). To increase their adaptive potential, haploid cells can also switch mating types through recombination with silent loci after an HO endonuclease cut (Haber, 2012).

One of the reasons supporting a sexual reproduction of *C. glabrata* is the existence and the observation of mating types a and α (Muller et al., 2008; Carreté et al., 2018). Even more striking, Brockert et al., 2003 reported mating type switching during infection of a host and Butler et al., 2004 reported mating type switching during laboratory growth. Interestingly, *C. glabrata* possesses a seemingly intact mating machinery in its genome (S. Wong et al., 2003), composed of orthologues of genes involved in mating in *S. cerevisiae* : *MTL1*, *MTL2* and *MTL3* genes were found in *C. glabrata* (Srikantha et al., 2003; Butler et al., 2004). *MTL1* is the expression locus, while *MTL2* and *MTL3* encodes a and α mating informations. The genome also contains an homothallic (HO) endonuclease, which is known to be responsible for gene conversion events that underlies mating type switching in *S. cerevisiae*. Additionally, Butler et al., 2004 found a putative HO endonuclease recognition site in *MTL1*.

All these findings were further confirmed by Carreté et al., 2018, who furthermore demonstrated the existence of illegal repair events during switching (Figure 1.5). They also found traces of selective constraints in mating genes. Even more interesting, they found the same levels of constraint in *C. glabrata* than in *S. cerevisiae* or *C. albicans*, which have functional sexual and parasexual cycles, respectively. This tends to show that sexual genes in *C. glabrata* are still under selective pressure, most likely because they are still functional. Also, switching is lethal to many *C. glabrata* cells in laboratories Boisnard et al.,

2015, which shows that the switch is tightly regulated. Besides, they discovered evidences of chimeric patterns in chromosomes, which is the result of sexual mating between yeasts of different clades.

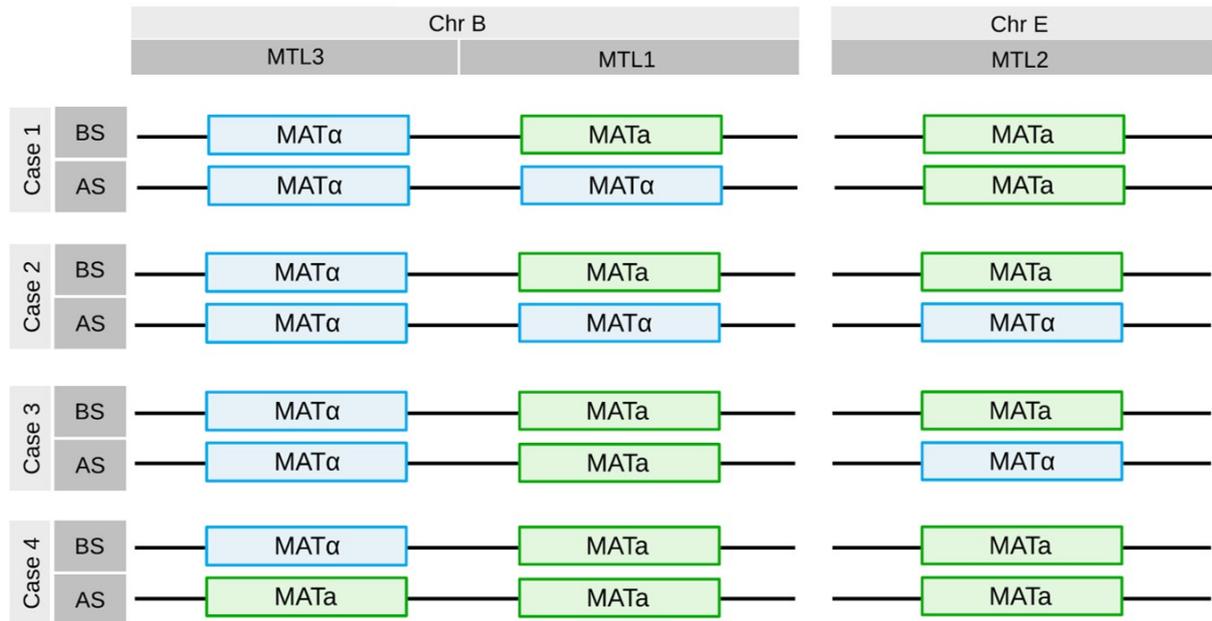


FIGURE 1.5. Example of illegal repair events during switching in *C. glabrata*. BS means Before Switching. AS means After Switching. Adapted from Carreté et al., 2018. Case 1 represents a normal conversion event at *MTL1*, which switched mating type from a to α . Case 2 shows the classic a-to- α switch at *MTL1* accompanied by illegitimate conversion at *MTL2*, resulting in a triple- α strain. Case 3 displays an illegitimate *MTL2* conversion without the *MTL1* switch, resulting in a triple-a strain.

To summarize, *Candida glabrata* keeps distinct a and α haploid mating types and distinct associated cellular identities (Muller et al., 2008) ; it shows evidences of mating type switches in populations issued of isolates and laboratory populations (Brockert et al., 2003; Butler et al., 2004) and sexual recombination (Dodgson et al., 2005; Carreté et al., 2018). All this lets us think that *C. glabrata* might still be mating but rarely and we just haven't found yet the adequate conditions to observe it.

***Candida glabrata* and the stress responses**

As mentioned in the previous part, one of the possible explanations of *C. glabrata* success as a human pathogen and a commensal of a lot of ecological niches could be its genome plasticity, among other things. This genome plasticity has been shown to allow the yeast to adapt to a range of environments, which can vary a lot in pH, temperature, oxygen and iron availability, oxidative stress. . . A brutal change in these conditions is perceived by the yeast as a stress and triggers several pathways that aim to adapt the cells to their new environment to improve their survival and growth. The phenomenon of adaptation to improve the cells fitness in a specific environment is called stress response. Besides genome plasticity, stress responses can also take the form of transcriptional adaptations inducing changes in gene expression and protein composition of the cell. We can distinguish two kinds of stress responses : a general stress response, which is activated as soon as the cells detect an environmental variation, regardless of the change, and stress responses specifically adapted to the triggers perceived by the cells.

2.1 A general response : the Environmental Stress Response (ESR)

The Environmental Stress Response (ESR) corresponds to the regulation of a core set of genes in response to sub-optimum growth conditions, regardless of the stress imposed. It means that the cells react to changes in the environment by up and down-regulating the same set of genes for every condition, whether it is temperature, pH or nutrients availability. . . On a more practical way, it also means that these genes will display close expression profiles during the stress response. The ESR was studied in *Candida glabrata* by Roetzer et al., 2008 through the yeast transcriptional response to four conditions : glucose starvation, osmotic stress (addition of NaCl in the media), heat stress (growth at 42°C) and oxidative stress (addition of hydrogen peroxide in the media). They identified 782 genes as members of the ESR. Among those, 358 were induced and 424 were repressed. Interestingly, the ESR seems to be triggered not only by deeply detrimental growth conditions, but also by small environmental changes which do

not severely impact cell growth or viability. Nevertheless, the ESR is caused by the transition from an optimal environment to a sub-optimal environment and therefore is not activated when cells go back from a sub-optimal environment to an optimal growth environment. It is equally noteworthy that the ESR intensity is adapted to the severity of the environmental stress : the more severe the environmental changes are, the longer the ESR is and the more acute are the genes induction and repression.

The genes composing the ESR are enriched in several functional categories. More specifically, the stress response causes the general repression of cytosolic translation (ribosome biogenesis, rRNA and tRNA processing, translation initiation and elongation), nucleotide biosynthesis, cell growth and secretion. On the other hand, it causes the induction of HSP chaperone proteins (as well as protein folding and degradation), cellular redox reactions, DNA damage repair and genes involved in energy storage and carbohydrate metabolism. In short, all these changes aim to protect DNA, save energy by repressing protein synthesis and cell growth and rearrange the proteome to keep the proteins essential for stress response and basic cell functioning. It gives rise to an interesting phenomenon : the simultaneous induction of both synthetic and catabolic enzymes, which aim to help produce the suitable proteins and destroy the useless ones. The ESR might also be the cause of cross-resistance to various stresses, in which cells exposed to a low dose of one stress become resistant to a low dose of a second stress : by protecting the pathways previously mentioned after a first stress, the cells are already in a resistance state which reduces the impact of a second stress.

Roetzer et al., 2008 also showed that Msn2/Msn4 TF are partially responsible for the ESR. Deletion of Msn2/4 abolishes the induction/repression of a part of the ESR and render cells more sensitive to stress exposure. These factors bind on the STRE motif (AGGGG) (discovered by Martínez-Pastor et al., 1996 in *S. cerevisiae*), which happens to be one of the most enriched motifs in ESR genes. Msn factors expression and localisation are tightly regulated. When cells encounter stresses, these TFs are induced, and Msn2/4 proteins are relocated from cytoplasm to nucleus to induce several genes of the ESR. When cells return to a more growth-friendly environment, Msn2/4 are down-regulated and they are exported from the nucleus. Thus, the import/export of these TF is essential for their regulation abilities. The mechanisms involved in nuclear import and export are not fully understood yet, but it is clear that the Nuclear Export Signal (NES) (especially the conserved HD1 domain) and the Nuclear Localisation Signal (NLS) play an important role. The NLS drives nuclear import. Nuclear export requires HD1 domain presence, as well as exportin Msn5. Currently, it is supposed that HD1 is the target site of Msn5 exportin and that the Protein Kinase A (PKA) phosphorylation site within HD1 has a regulatory role.

Msn2 activity is likely regulated by PKA and TOR pathways, which phosphorylate the HD1 domain and the NLS, but these assumptions remain to be proven in *C. glabrata*.

These findings differ only slightly from the ESR study of *S. cerevisiae* (Gasch et al., 2000). They used 13 different stress conditions and found that the core set of genes contains 868 genes, among which 283 are upregulated and 585 are downregulated. In both *C. glabrata* and *S. cerevisiae*, ESR genes represent 15% of the total number of genes in the genome. Several dozens of the ESR genes are common between the two species (Fig 2.1). As a consequence, there is an enrichment in the same GO categories in both species (see previous paragraphs). However, there are still a few differences in the targets : genes of sterol and ergosterol biosynthesis are deeply repressed upon stress in *C. glabrata* while they are not in *S. cerevisiae*. Some of these genes were showed to be linked with cell growth and drug resistance (Geber et al., 1995; Montañés et al., 2011), which might explain their down-regulation during the ESR. Additionally, some interesting genes are up-regulated in *C. glabrata* and down-regulated in *S. cerevisiae* : *PHO84* (involved in heavy metal tolerance, Rosenfeld et al., 2010), *VPH2* (involved in vacuolar pH and lifespan extension, Ruckenstuhl et al., 2014), *TBF1* (TF involved in DNA protection, Ribaud et al., 2012). Interestingly, Wapinski et al., 2010 showed that *C. glabrata* lost the down-regulation of ribosomal proteins in ESR, while it is conserved from *S. cerevisiae* to *C. albicans*.

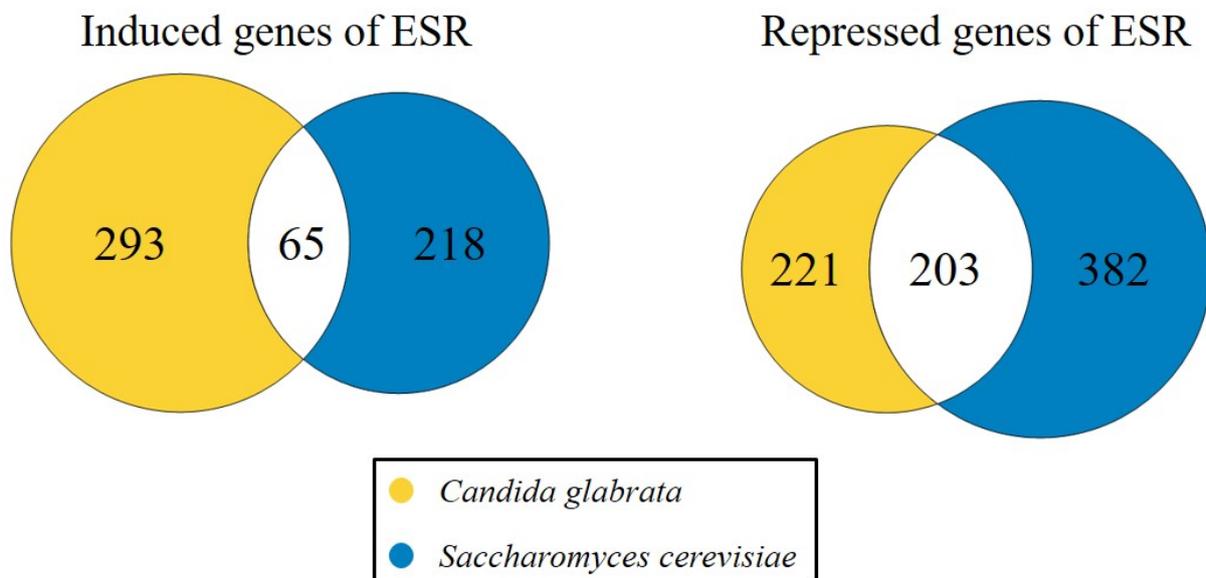


FIGURE 2.1. ESR genes in *S. cerevisiae* and *C. glabrata* genomes. The overlap between the ESR of the two species is depicted as a Venn diagram. *C. glabrata* and *S. cerevisiae* data are from Roetzer et al., 2008; Gasch et al., 2000, respectively.

The expression of ESR genes in *S. cerevisiae* is regulated by different TF, depending on the environmental condition encountered, and governed by several different upstream signaling pathways. Msn2 general role and localisation are especially conserved in *S. cerevisiae* (Görner et al., 1998; Görner et al., 2002). Other pathways are involved, and it seems that each stress condition activates specific pathways. Notably :

- PKA and PKC pathways trigger the ESR (especially repression of ribosomal proteins) in response to lack of nutrients and impaired secretion, respectively (Klein et al., 1994; Neuman-Silberberg et al., 1995; Nierras et al., 1999).
- ESR-induced genes during osmotic stress are regulated by the High Osmolarity Glycerol (HOG) pathway (Rep et al., 2000).
- ESR-triggering by DNA damage is controlled by DNA damage specific Mec1 pathway (Gasch et al., 2001).

Unfortunately, most of these transcription factors and pathways are poorly studied in *C. glabrata*. The PKA pathway seems to be involved in adaptation to nutrient deprivation and biofilm formation (Schwarz Müller et al., 2014; d'Enfert et al., 2016), but it wasn't proven to be involved in ESR regulation. The PKC pathway plays a role in response to cell wall damaging agents, however it wasn't linked to the ESR (Borah et al., 2011). Schwarz Müller et al., 2014 noted that Mec1 isn't essential in *C. glabrata* while it is in *S. cerevisiae* and that its deletion modifies the cells fitness which suggests that the Mec1 regulatory networks functions differently between the two species, but once again, no link was made with the ESR. Nevertheless, Gregori et al., 2007 and Roetzer et al., 2008 showed that Hog1 function is conserved in *C. glabrata* and it retains its role in ESR, particularly during osmotic stress.

A part of the ESR genes is also conserved in other species such as *L. kluyveri* (Brion et al., 2016), *S. pombe* (D. Chen et al., 2003), *Aspergillus spp* (Kawasaki et al., 2002; Du et al., 2006). Usually, the target genes in these species have GO categories similar to those of *S. cerevisiae* and *C. glabrata*. The ESR existence is still unclear in *C. albicans* because of contradictory findings (Enjalbert et al., 2003; Marcus et al., 2004; Enjalbert et al., 2006; Roy et al., 2013), even if at least a small part of the regulations seems to be conserved.

Regarding the role of Msn2, there are contradictory signs in previously mentioned species. The Msn2 DNA binding domain is found in genes in other species, but this is usually the only conserved part

between these genes and *C. glabrata* Msn2. A team found STRE binding proteins in *S. pombe* (Kunitomo et al., 2000), however they didn't seem to be involved in stress response. Nevertheless, the HD1 domain is conserved in *K. lactis* and *A. gossypii*, as well as the embedded PKA phosphorylation site and is enough to trigger nuclear export of Msn2 in these species. However, the HD1 region doesn't appear to be conserved in *C. albicans* and its close species, which led Roetzer et al., 2008 to hypothesise that the HD1 domain and the Msn2-mediated stress response appeared after the divergence of *C. albicans* from the evolutionary path leading to *S. cerevisiae* and *C. glabrata*. Enjalbert et al., 2006 found no role for Msn2 in *C. albicans* ESR. Ramsdale et al., 2008 showed that *C. albicans* Mnl1 (Msn2 correspondent in this yeast) is still binding to STRE motifs, however, they only reported its role in weak-acid response, and not in a larger environmental stress response. On the other hand, Alonso-Monge et al., 1999 reported an important role for Hog1 in several types of stress in *C. albicans* (osmotic stress, weak acid stress, oxidative stress). Also, it was shown by D. Chen et al., 2003, that Sty1 (Hog1 *S. cerevisiae* orthologue in *S. pombe*) is required for ESR regulation in *S. pombe*. Hence, the ESR of these two species might be carried out by transcription factors that are different from Msn2 orthologues.

Besides the control of the ESR, these signaling systems have also been involved in regulating more specialized stress responses and the associated gene expression. These pathways simultaneously regulate both the ESR and specialized responses specifically adapted to the environmental conditions that activate the pathways.

2.2 Condition-specific stress responses

Like most unicellular organisms, *Candida glabrata* has a multitude of ways to deal with ever-changing growth conditions or unfavorable media and nutrients starvation. These situations are perceived by the cells as stresses and trigger stress responses specifically adapted to answer the changes in growth media. As a result, *C. glabrata* has a lot of different stress response pathways. In the following parts, a handful of them will be addressed, as well as the strong connections that exist between them : the absence of oxygen, the oxidative stress response, the respiratory pathway and iron homeostasis. We focused on these stresses because they are of interest to our team and because some of them (oxidative stress, changes in iron concentration) are important for *C. glabrata* virulence.

2.2.1 Rox1, a mediator of response to hypoxia

ROX1 was extensively studied in *S. cerevisiae* since the early 80's. It was first discovered as a repressor of *ANBI*, a translation elongation factor especially expressed during hypoxia (Lowry et al., 1986). Lowry et al., 1988 confirmed the repressing effect of Rox1 on another gene involved in hypoxia, *CYC7*, and Ter Linde et al., 2002 and others used genome-wide approaches to show that under aerobic media, Rox1 represses several tens of genes required for hypoxia transition and adaptation. This regulation is permitted by Rox1 binding on the ATTGTTCTC motif found in the promoters of its targets (Balasubramanian et al., 1993). The binding is mediated by HMG (High Mobility Group) domain. Deckert et al., 1995 also found that Rox1 ability to repress its targets was probably linked to its DNA bending capacities : when Rox1 is bound, it bends DNA at an angle of 90 degrees, preventing other activators or polymerases to bind and promote or start transcription. Currently, *ROX1* is almost uncharacterised in *C. glabrata*. Schwarzmüller et al., 2014 reported no impact of Rox1 deletion on *C. glabrata* fitness in the condition they tested (azole susceptibility and biofilm formation). However, Gupta et al., 2017 showed that Rox1 has a role in biofilm formation and biofilm resistance to azole under hypoxic condition. Additionally, they suggested that Rox1 is involved in resistance to hypoxia. This last role resembles a lot to the role of *S. cerevisiae* Rox1.

However, in *S. cerevisiae*, Rox1 activity is also mediated by its interactions with several other proteins (Kastaniotis et al., 2000; Sertil et al., 2003; Mennella et al., 2003; Klinkenberg et al., 2005). Rox1 interacts synergistically with Mot3 to repress the transcription of hypoxic genes, and that Mot3 activity is regulated by oxygen, like Rox1. It is also able to recruit the Tup1/Ssn6 complex, a known general transcriptional repressor, to repress its targets.

With all these data, the ability of Rox1 to regulate its targets was also rapidly linked to the presence of oxygen and heme : Lowry et al., 1988 and L. Zhang et al., 1999 showed that *ROX1* is induced by heme, and this finding laid the bases of *ROX1* regulatory network. Indeed, Keng, 1992 demonstrated that Heme Activated Protein 1, Hap1, was required to induce *ROX1* to regulate *HEM13*. Zitomer et al., 1997 went further and found that the presence of heme induces Hap1 which in turns induces Rox1 which represses his targets. On the other way, absence of heme causes *ROX1* repression by Hap1, thus lifting Rox1 repression on hypoxia genes. This makes sense, given that heme biosynthesis is strongly linked to oxygen availability. Also, according to Proft et al., 2005, Sko1 is another possible regulator of *ROX1*, as well as Ixr1 (Castro-Prego et al., 2009).

Besides its regulation by heme and oxygen and its impact on hypoxic genes, *S. cerevisiae* Rox1 was found to be involved in a variety of roles, such as lipid metabolism (Vasconcelles et al., 2001; Kwast et al., 2002), cell wall mannoproteins regulation (Abramova et al., 2001), ergosterol biosynthesis regulation with Hog1 during osmotic stress (Jensen-Pergakes et al., 2001; Montañés et al., 2011) and fermentation (Fujiwara et al., 1999). More interestingly, Rox1 was also linked to metal homeostasis and oxidative stress response, which is not so surprising given the relationships between oxygen and ROS production : C.-M. Wong et al., 2003 showed that Rox1 regulates *TSA2*, a key gene of the OSR. Liu et al., 2013 determined that oxidative stress lowers the occupancy of Rox1 on hypoxic gene promoters, thus inducing their de-repression. Finally, Caetano et al., 2015 confirmed that during oxidative stress caused by cadmium, Yap1 activates *ROX1* to repress *FET4*, providing a link between Rox1, OSR and metals. This link might be conserved in *C. glabrata*, because Merhej et al., 2016 found that Yap1 binds and regulates *ROX1* in response to selenite, which causes oxidative stress, among others. Still in *C. glabrata*, Rox1 has another role in metal homeostasis : Srikantha et al., 2005 showed that it regulates *CCC2*, a copper transporter. This regulation is conserved in *S. cerevisiae* (Ter Linde et al., 2002). Also, *C. glabrata* Rox1 was linked to mitochondrial dysfunction (Ferrari et al., 2011b). Nevertheless, the role of Rox1 doesn't seem to be conserved in *K. lactis* (Fang et al., 2009) nor in *C. albicans*, where it controls filamentous growth (Khalaf et al., 2001; Kadosh et al., 2001).

2.2.2 The oxidative stress response (OSR)

Oxidative stress occurs when the concentration of Reactive Oxygen Species (ROS) present in or around the cell is higher than the detoxification abilities of the yeast or when damages resulting from ROS accumulation starts proving difficult to repair. ROS are highly reactive chemical species containing oxygen, such as the superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (HO^{\cdot}). These compounds can damage DNA, oxidize proteins and lipids and ultimately cause cell death. There are several sources of ROS (reviewed in D'Autréaux et al., 2007). First, they are by-products of normal aerobic metabolism, especially cellular respiration : for example, they are formed during oxidative phosphorylation, which aims to produce ATP. Additionally, hydroxyl radicals and superoxide anions are produced during Fenton reactions, in which metallic ions (which are often iron and copper, see Stohs et al., 1995) in contact with hydrogen peroxide are oxidized, leading to the formation of the previously mentioned species. Heavy metals such as cadmium and lead can also cause oxidative stress by peroxidising lipids and oxidising glutathiones (Stohs et al., 1995; Z.-S. Li et al., 1997). The damages caused

by oxidation of proteins and lipids has a great cost in ATP and NADP(H) for the cells, because the reduction processes of these compounds are very expensive in energy. Finally, pathogenic yeasts such as *C. glabrata* are likely to come into contact with ROS when they are engulfed by macrophages during the systemic invasion of a host. Phagocytes are the first line of host defense against fungal infections and when they encounter a pathogen, they quickly use the NADPH oxidase complex to produce ROS designed to kill it (Segal et al., 2012), such as, for example, hypochlorous acid, the main reactive component of bleach. Failing in NADPH oxidase functioning of the host often leads to increased susceptibility to fungal infections, which displays a strong link between oxidative stress and virulence (Roetzer et al., 2011b; Seider et al., 2014). This shows how important it is for *C. glabrata* to efficiently deal with ROS and maintain a proper redox homeostasis.

It was also shown that the Oxidative Stress Response (OSR) plays a key role in *C. glabrata* survival and replication after engulfment in macrophages (Kaur et al., 2007; Wellington et al., 2009; Roetzer et al., 2010; Roetzer et al., 2011b; Seider et al., 2011). The OSR is so efficient in *C. glabrata* that this yeast can withstand very high H₂O₂ concentrations : while the H₂O₂ concentration inside a macrophage is 0.4 mM, *C. glabrata* can resist up to 40 mM H₂O₂ given proper conditions (Roetzer et al., 2010), which might partially explain its persistence in these immune cells. Studies showed that *C. glabrata* is particularly resistant to hydrogen peroxide, compared to *S. cerevisiae* and isolates of *C. albicans* (Cuéllar-Cruz et al., 2008), even if OSR is quite conserved between *S. cerevisiae* and *C. glabrata* (Roetzer et al., 2008; Salin et al., 2008; Saijo et al., 2010; Roetzer et al., 2011b; Gutiérrez-Escobedo et al., 2013). Cuéllar-Cruz et al., 2008 also proved that *C. glabrata* ROS resistance is further increased in stationary phase.

The currently available data suggest that *C. glabrata* has a robust and most likely redundant antioxidant system. The OSR in *C. glabrata* is performed through the induction of enzymatic (catalases, SODs, and peroxidases) and non-enzymatic (glutathione) mechanisms. It will mainly use three pathways to deal with ROS and especially hydrogen peroxide.

- The catalase pathway (Green box in Figure 2.2) : catalases decompose H₂O₂ to water and oxygen. While *S. cerevisiae* has two catalases, *CTT1* (cytoplasmic catalase) and *CTA1* (peroxisomal catalase), *C. glabrata* has only one, *CTA1*. Interestingly, *CgCTA1* combines features (induction conditions and intracellular localisation) of the two catalases of *S. cerevisiae* (Roetzer et al., 2010; Roetzer et al., 2011b). Even if *CTA1* orthologue of *C. albicans* (*CAT1*) was proved to be important for virulence (Nakagawa et al., 2003), that is not the case in *C. glabrata*, despite being essential to hydrogen peroxide resistance (Cuéllar-Cruz et al., 2008; Roetzer et al., 2010).

- The glutathione pathway (Blue box in Figure 2.2) : it is constituted by glutathione (GSH), glutaredoxins and a glutathione reductase. This system uses a series of reactions that are expensive in energy and reduced materials. Glutathione (GSH) is an essential tripeptide composed of glycine, cysteine and glutamate synthesized by the action of *GSH1* and *GSH2* (Meister, 1974). In *C. glabrata*, *GSH1* is essential (Yadav et al., 2011). GSH acts as an electron donor for conversion of H_2O_2 into water. Accordingly, it produces an oxidised form of GSH, glutathione disulfide (GSSG) (Penninckx, 2000). This reaction is catalysed by the glutathione peroxidase enzymes Gpx1 and Gpx2. GSH is reformed by reduction of GSSG by the glutathione reductase, Glr1, using NADPH as an electron donor. The glutaredoxins Grx1 and Grx2 may also be involved in the maintenance of a high GSH to GSSG ratio.
- The thioredoxin pathway (Red box in Figure 2.2) : this system also uses a series of complex and expensive reactions. Detection of ROS leads to the activation of *TRR1*, a reductase which consumes NADPH to reduce the thioredoxin Trx2. Trx2 then reduces the thioredoxin peroxidase Tsa1, which converts H_2O_2 to H_2O . In the same reaction, Tsa1 is also reduced back to its initial form (Grant, 2001). In *S. cerevisiae*, Tsa1 is expressed at high basal levels even in unstressed cells. This might be because it is an essential antioxidant of ROS (its deletion renders cells sensitive to a range of oxidative stressors) but also because Tsa1 was linked to genome stability and protection (Iraqi et al., 2009).

Furthermore, superoxides anions and hydroxyl radicals are converted to hydrogen peroxide by superoxide dismutases (SOD) (Briones-Martin-Del-Campo et al., 2014). This allows the anions to enter one of the three recycling pathways mentioned above. SODs are metalloenzymes that catalyze the dismutation of $O_2^{\cdot-}$ into H_2O_2 and oxygen. SODs always have a metal cofactor, which can be copper, zinc, iron, manganese... *C. glabrata* has two SOD genes, *SOD1* (Copper and Zinc SOD) and *SOD2* (Manganese SOD). *SOD1* and *SOD2* are constitutively expressed, even in the absence of oxidative stress (Roetzer et al., 2011b), contrary to *S. cerevisiae*, and are also highly induced in glucose starvation. Unlike in *C. albicans*, sole deletion of *SOD1* doesn't impact survival in macrophages (Roetzer et al., 2011b).

Additionally, *C. glabrata* can reduce the impact of heavy metals with the use of chelator proteins called metallothioneins. Two of them are identified in this yeast : *MT-I* (*CAGL0D01265g*) and *MT-II* (*CAGL0H04257g*) (Mehra et al., 1989), however their functioning is still unclear. Mehra et al., 1994 also demonstrated the use of phytochelatins (GSH derivatives) to chelate cadmium. Finally, pigments also

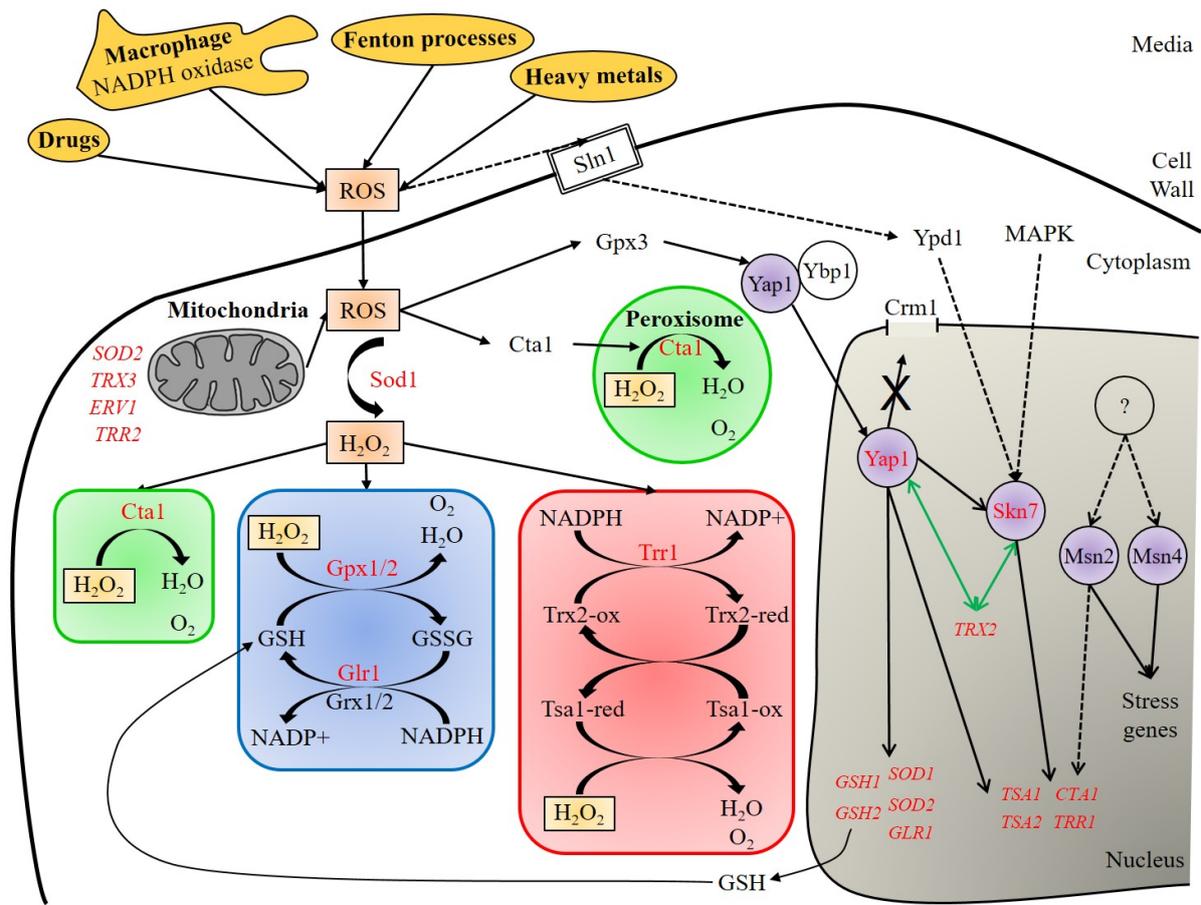


FIGURE 2.2. Pathways of the OSR in *C. glabrata*. Drugs involve for example Menadione, a known cause of $O_2^{\cdot -}$ production, or benomyl. Fenton processes are oxidation reactions between hydrogen peroxide and metals, such as iron. Heavy metals can be Cadmium ions, for example. ROS and ROS producers are in orange boxes and orange drawing respectively. The three main H_2O_2 detoxification pathways are also boxed : catalase (green, in cytoplasm and peroxisomes), glutathione (blue) and thioredoxin (red). TFs are in violet. The genes identified as transcriptionally regulated by oxidative stress and Yap1 are written in red. The conformational change of Yap1 which prevents it from leaving the nucleus is represented by a black cross. Curved arrows are redox reactions. Green double-ended arrows imply a positive feedback. Dashed arrows represent putative pathways.

play a role against H_2O_2 protection : Brunke et al., 2010 showed that a tryptophan-derived pigment is a by-product of the Ehrlich pathway of tryptophan degradation and its production is mainly driven by the aromatic aminotransferase I (Aro8).

The OSR in *C. glabrata* is mainly governed by four transcription factors, which have redundant targets. These information are summarised in Figure 2.2. The first TFs are Msn2 and Msn4, which were previously mentioned for their importance in the ESR. The most striking gene under their control is *CTA1*. Additionally, Cuéllar-Cruz et al., 2008 showed that Msn2 is important for resistance to ROS especially in stationary phase, while Msn4 is essential in both stationary phase and log-phase.

The third factor involved in OSR regulation is Skn7. Skn7 was originally isolated as a high-copy

number suppressor of a strain defective in β -glucan synthesis : J.L. Brown et al., 1993 revealed that Skn7 was controlling *KRE9*, a gene encoding for a glycoprotein involved in cell wall beta-glucan assembly. Several teams confirmed that result and found other target genes involved in the same pathways, namely osmotic stress response and cell wall regulation.

Afterwards, a huge focus was made to understand Skn7 activation upon osmotic stress. In particular, Ketela et al., 1998; Lu et al., 2003; Lu et al., 2004; Shankarnarayan et al., 2008 deciphered the mechanism of Skn7 activation. It starts with the detection of osmotic stress by the sensor kinase module Sln1. This event provokes the auto-phosphorylation of Sln1 and the transfer of the phosphorylated group from Sln1 to Ypd1. This last protein is a shuttle that will go into the nucleus to interact with Skn7, which it activates by transfer of the phosphorylated group with the help of Mog1. After its activation, Skn7 will regulate its targets and contribute to the regulation of the HOG pathways, the main pathway to respond to osmotic stress. In parallel, Skn7 can also be activated by the plasma membrane-associated cell wall sensor *MID2* (Ketela et al., 1999). Interestingly, *MID2* is known to interact with genes required for cell wall construction and cell wall integrity signalling, and deletion of *MID2* leads to resistance to calcofluor white, an agent attacking the fungal cell wall and triggering the osmotic response.

The osmotic stress function of Skn7 might be conserved in *C. glabrata* : Juárez-Cepeda et al., 2015 showed that the *EPA2* adhesin encoding gene is responsive to stress and that its activation relies on Skn7, the *EPA* genes being well-known for their role in biofilm formation. However, it seems that this function is lost in *C. albicans* : Basso et al., 2017 reported that in *C. albicans*, Skn7 lost its role in cell wall regulation and osmotic stress response, but acquired the ability to regulate filamentation and morphogenesis.

In parallel, Skn7 was also found to be involved in the oxidative stress response in *S. cerevisiae* (Morgan et al., 1997; Lee et al., 1999; Tsuzi et al., 2004). This role is conserved in *C. glabrata* : Skn7 is responsible for the induction of genes critical to the OSR such as *GPX2*, *TRX2*, *TRR1*, *TRR2*, *TSA1*, *TSA2* or *CTA1* (Cuéllar-Cruz et al., 2008; Saijo et al., 2010). Interestingly, Skn7 is constitutively localised in the nucleus in *C. glabrata* (Roetzer et al., 2011b), so its activation during stress doesn't rely on cellular localisation. Eventhough, it is still unclear how Skn7 is activated, Gómez-Pastor et al., 2013 showed in *S. cerevisiae* that Trx2p regulates Skn7p phosphorylation and thus modulates Skn7p-dependent promoter activation during oxidative stress. This displays a clear link with the fact that Skn7 has to be phosphorylated to activate antioxidant genes (He et al., 2009). Given that both Skn7 and Trx2

are conserved in *C. glabrata* and that Skn7 still regulates *TRX2*, it is possible that phosphorylation may also play a role in Skn7 activation in this species. Additionally, Skn7 might also be activated by the Ras/PKA and MAPK pathways (Charizanis et al., 1999). However, these mechanisms weren't studied in *C. glabrata*. The role of Skn7 as a regulator of oxidative stress is conserved across many species of fungi, including *C. albicans* (Homann et al., 2009; Basso et al., 2017), *A. fumigatus* (Lamarre et al., 2007) and *C. neoformans* (K.-W. Jung et al., 2015). The conservation of the Skn7 regulatory network across several species is reviewed in Pais et al., 2016.

Additionally, other roles were associated to Skn7 in *S.cerevisiae* : it is involved in recombinational repair of double-strand breaks in DNA by controlling Rad51 with the help of Yap1 (Yi et al., 2016). It might also be involved in heat shock response, given that it can interact with Hsf1 to activate heat shock genes (Raitt et al., 2000) and that cells deleted for Skn7 are more sensitive to heat Morano et al., 2012. Also, Skn7 is often considered as a Heat Shock Factor-like TF, because it shares structural homologies with Hsf1 (Raitt et al., 2000) and both Skn7 and Hsf1 bind the same DNA motif, namely TTCnnGAAnnTTC (Raitt et al., 2000 and Amin et al., 1988). In *C. glabrata*, Skn7 is also linked to virulence : Saijo et al., 2010 showed that cells deleted for Skn7 present an attenuated virulence in murine model. It implies that this factor or its targets are likely to be involved in virulence processes.

Finally, in *C. glabrata*, most of the genes mentioned here, including Skn7, are also co-regulated by Yap1 (Merhej et al., 2016), the last factor that will be mentioned here and the most important in term of transcriptional regulation. It has been shown that Yap1 and Skn7 have independent roles in regulating oxidative stress adaptation, but also co-operate to regulate many genes by co-binding the same promoters, and this is similar to *S. cerevisiae* (Roetzer et al., 2011b). Moreover, in *S. cerevisiae*, Skn7 has to be phosphorylated to physically interact with Yap1 (Mulford et al., 2011). In *C. glabrata*, contrary to Skn7, Yap1 is essential to the OSR, but not in virulence (Cuéllar-Cruz et al., 2008; K.-H. Chen et al., 2007). Upon oxidative stress, Yap1 is translocated from the cytoplasm to the nucleus and phosphorylated in *S. cerevisiae* : Gpx3 is a key sensor of oxidative stress, and when it is reduced, it changes Yap1 conformation and especially its NES, preventing its export of the nucleus by Crm1 (Yan et al., 1998), which leads to Yap1 accumulation in the nucleus. While there is no effective clue on Gpx3 role in OSR in *C. glabrata*, Yap1 functional domains (NLS, NES and Gpx3 interaction domain) are conserved, so this mechanism might also be conserved. Indeed, Roetzer et al., 2010 showed that Yap1 is localised in the nucleus after oxidative treatment. Once in the nucleus, the bZIP TF Yap1 will regulate a wide range of targets, including genes in oxidative stress and redox homeostasis (previously

mentioned genes and also GSH genes, for example), drug resistance (K.-H. Chen et al., 2007), cadmium resistance, iron metabolism, heme biosynthesis... (Merhej et al., 2016). It will do so by binding mainly the YRE-O motif TTACTAA (K.-H. Chen et al., 2007; Lelandais et al., 2008; Merhej et al., 2016), even if other binding motifs were reported (Lelandais et al., 2008; Tan et al., 2008; Kuo et al., 2010; Goudot et al., 2011), notably the YRE-A motif TTACGTAA. It is also interesting to note that Yap1 and its role in oxidative stress are very conserved across fungi (Enjalbert et al., 2006; Wang et al., 2006; Lessing et al., 2007; Leal et al., 2012; Paul et al., 2015).

If we take into account all these studies, we can note that the OSR in *Candida glabrata* has some interesting global features : The regulation of genes involved in oxidative stress is dose dependent in *C. glabrata* (Puttnam, 2012) : the more acute the stress is, the more genes are up/down-regulated and the more intense the expression variations are. It also varies with the length of the stress imposed to the cells. The comparison of the results of Lelandais et al., 2008 and Roetzer et al., 2010 also shows that the transcriptional regulation is conditioned by the oxidative stressor used : benomyl, menadione and H₂O₂ don't exactly elicit the same response. Finally, given the genes involved in the OSR, the stress response appears to be strongly related to cellular respiration and iron homeostasis (Merhej et al., 2016). Indeed, many genes induced in response to oxidative stress are also involved in metal ion homeostasis (see Introduction Chapter 3).

2.2.3 The CCAAT-Binding Complex as a link between oxidative stress and respiration

Over the years, several teams identified other factors involved in oxidative stress, but one especially stood out from the crowd : Hap4. It was noteworthy for several reasons. First, in *S. cerevisiae*, Hap4 was found to be involved in the regulation of genes belonging to the OSR, such as *SOD1* (Chevtzoff et al., 2010), *SOD2* (Pinkham et al., 1997) and *TSA2* (C.-M. Wong et al., 2003). Second, Petryk et al., 2014 suggested that Hap4 and Yap1, the key regulator of the OSR, diverged from a common ancestor, which might suggest common regulating features. Finally, still in *S. cerevisiae*, Chevtzoff et al., 2010 showed that ROS regulate Hap4 activity and that Hap4 quantity plays an important role in the reduction of mitochondria biogenesis induced by ROS. All of this tends to display a strong link between ROS, the subsequent OSR, mitochondria and thus respiration. However, Hap4 is much better known for its huge role in regulation of cellular respiration alongside the CCAAT Binding Complex (CBC), reviewed in Bolotin-Fukuhara, 2017. Given that literature on CBC is quite restricted in *C. glabrata*, most of the

following information is inferred from *S. cerevisiae*, which proved to be a close species with shared features.

The CBC is a heterotrimeric complex which is widely conserved from yeast to plants and mammals, being named NF-Y A/B/C in the latter and Hap2/3/5 in the former. The HAP complex was discovered in *S. cerevisiae* in the early 80's by Guarente's team, who studied the regulation of the *CYC1* gene, coding for isocytochrome c, a protein of the mitochondrial respiratory chain. (Guarente et al., 1983) found an "enhancer" region responding to heme presence in the upstream sequence of *CYC1*. They later looked for trans-acting mutation affecting this region and found a mutant (Guarente et al., 1984) corresponding to the first member of the CBC, which was named *HAP2* (Pinkham et al., 1985). At that time, this group already noticed a link between *HAP2* and growth on non-fermentable carbon sources, as well as the likely ability of Hap2 to bind on DNA (Pinkham et al., 1987). The identification of the second member of the CBC, *HAP3*, was done by the same team in the same way a few years later (Hahn et al., 1988). Interestingly, they also showed that Hap2 and Hap3 were interacting in a complex, which formation was sensitive to the carbon source (Olesen et al., 1987), and they found a common binding motif on DNA for both factors : CCAAT, hence the name of the complex. The third and last member of the CBC, Hap5, was identified by McNabb et al., 1995 using a yeast two-hybrid screen. Incidentally, it shared the same binding motif and the same carbon source phenotype than Hap2 and Hap3. This team also demonstrated that the CBC (Hap2/3/5) can bind to DNA without any other protein association. Although being essential to growth in non-fermentable carbon source, none of these proteins are actually transcriptionally induced by such a growth media. Orthologues of the three proteins are present in *C. glabrata*. In the mean time, several studies also confirmed that the Hap factors were highly homologous with human and mouse proteins and shared the same DNA binding domain. It was even showed that heterologous complex are steady (Becker et al., 1991).

Interestingly, Forsburg et al., 1989 identified a last gene, called *HAP4*, using the same method than Hap2 and Hap3. Despite not resembling the other CBC proteins, not being required for CBC binding on DNA, and not directly binding the CCAAT motif on DNA, it has the same carbon source phenotype (unable to grow on non-fermentable carbon source). The fact that CBC binding by itself isn't enough to provoke a transcriptional regulation and that *HAP4* is transcriptionally induced on non-fermentable media, contrary to *HAP2/3/5* implies that Hap4 is an activator part of the complex. All domains of interest found in the Hap proteins are represented in Figure 2.3. Hap4 clearly displays an activator domain, contrary to Hap2/3/5. Noteworthy, Hap4 proteins were only found in fungi (including *C. glabrata*) and

never identified in the CCAAT-binding proteins of mammals (Mantovani, 1999), which correlates with the fact that NFY complex alone can both bind DNA and activate transcription.

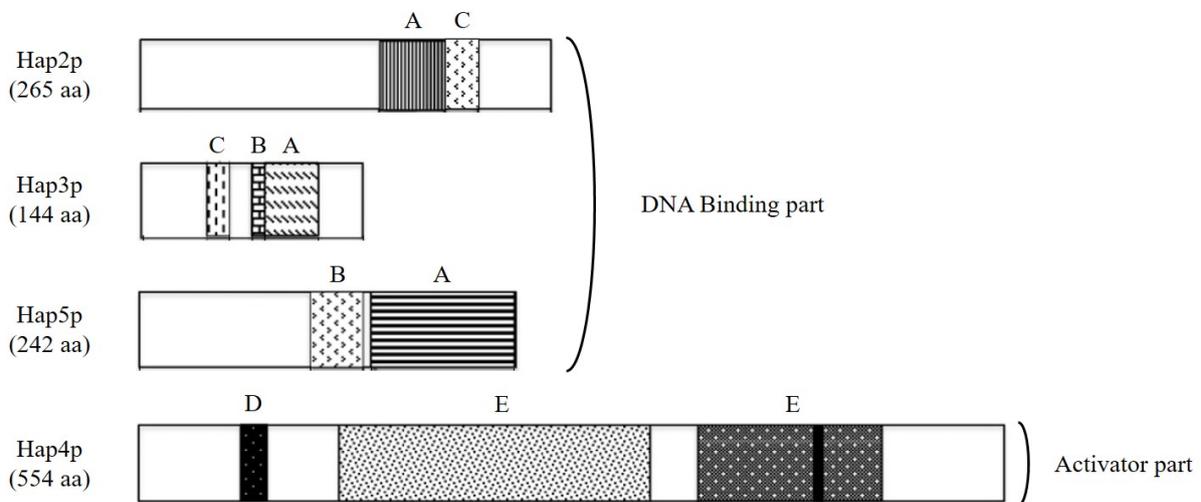


FIGURE 2.3. Representation of the four proteins of the HAP complex in *S. cerevisiae*. This figure is adapted from Bolotin-Fukuhara, 2017. A represents domains of interaction between the subunits of the CBC. B represents domains of interaction with Hap4. C represents DNA binding domain. D represents domain of interaction between the CBC and Hap4. E represents activation domains of Hap4. The subunits Hap2, Hap3 and Hap5 first form an heterotrimer. It will bind to Hap4 mainly via the Hap5 recruitment domain, provided the DNA-binding sequence CCAAT is present (McNabb et al., 2005).

The study of the CBC targets began simultaneously with the discovery of the members of the complex. Amongst the first targets identified were *HEM1* (Keng et al., 1987), involved in heme biosynthesis, *COX* genes (Trueblood et al., 1988; Trawick et al., 1989) which constitute the cytochrome c oxidase enzyme (the fourth complex of the respiratory chain) and *CYT1* (Schneider et al., 1991) which belongs to the third complex of the respiratory chain. These targets and the previous data suggested a strong link between the Hap proteins and the regulation of genes of the respiratory metabolism. This was further validated by the identification of other genes under CBC regulation through promoter dissection and low-throughput molecular approaches : *KGD1* (Repetto et al., 1989), *HEM3* (Keng et al., 1992), *CIT1* (Rosenkrantz et al., 1994)... Finally, genome wide studies with microarrays allowed to confirm this and to identify hundreds of genes targeted by the CBC. For example, Murphy et al., 2015 studied the impact of Hap4 overexpression in glucose and found that it mostly induced mitochondria and TCA functioning and biogenesis as well as ribosomal proteins biogenesis. The overexpression even brought the cells in a state of respiratory energy production despite the availability of glucose for fermentation. Buschlen et al., 2003 performed the opposite experiment by deleting Hap2 and Hap4 to compare the expression with WT cells. They were able to add a few roles for the CBC, including induction of components of the mitochondrial translation apparatus, induction of genes related to mitochondrial proteins folding and

import as well as mitochondrial carriers. They noticed that most of these genes (and especially the respiratory chain genes) contain at least one CCAAT motif in their promoter. A large proportion of these targets seems to be conserved in *C. glabrata* (see Results and discussion 5).

The Hap activation of these genes relies on the necessity to activate respiration, for example when glucose is not available. It also depends on the growth rate of the cells. Basically, when glucose is scarce and growth drops below a certain limit, Hap4 is induced and the CBC activates the aforementioned targets (Raghevendran et al., 2006; Fendt et al., 2010). This regulation seems to be correlated with the needs of the cells : the more they need the respiratory pathway, the stronger Hap4 is induced. It means that Hap4 will be totally repressed during fermentation, mildly activated during diauxic shift and even more during full-respiratory growth. Other causes of activation of the Hap complex, when it is in charge of respiration activation, include alteration of chromatin structure (Galdieri et al., 2016), increase in heme biosynthesis (T. Zhang et al., 2017) and cellular glutathione redox state (Yoboue et al., 2012).

However, the high-throughput analyses also widened the scope of the CBC targets to non-respiratory chain related genes such as *FBP1* and *PCK1* (Mercado et al., 1992), key genes of the gluconeogenesis, *RPM2*, a mitochondrial RNase P, *AAC2* (Betina et al., 1995), an ADP/ATP carrier, *IME1* (Purnapatre et al., 2002), a meiosis regulator and *ASNI/GDH1*, two enzymes involved in nitrogen metabolism (Riego et al., 2002; Hernández et al., 2011). Buschlen et al., 2003 also found genes involved in oxidative stress (most likely to respond to the ROS produced during respiration), amino acids biosynthesis, cell morphology, mating, drug resistance, rRNA metabolism, sterol metabolism and pseudohyphal growth. Hap4 was even linked to apoptosis (Leadsham et al., 2010). All these findings are in accordance with the numerous phenotypes that were associated to mutations in the CBC and Hap4 (Bolotin-Fukuhara, 2017). With its essential roles in respiration and seemingly in other pathways, the CBC ought to interact with a lot of other transcription factors to coordinate the required transcriptional responses. For example, studies of the upstream region of Hap4 revealed putative binding sites for several regulators such as *GLN3*, *GCR1*, *GIS1*, *RGT1*, *MOT3*... All of which are known to be involved in functions proven to be associated with the HAP complex (for example, glucose and nitrogen metabolism, cell cycle...). Unfortunately, there is only a few concrete proofs of TFs interacting with the CBC. Lundin et al., 1994 identified a strong Mig1 (a glucose repressor) binding site in Hap4 promoter, however, Mig1 deletion didn't impact Hap4 expression. Roberts et al., 2009 found that Rsf1 is induced in respiration on non-fermentative media (namely, glycerol) and binds several genes associated to Hap4 but there is no direct connection between the two TFs. Brons et al., 2002 found a connection between Hap4 and Cat8, a

gluconeogenic TF, where deletion of one factor deeply affects the expression of the other; as well as the subsequent target genes but weren't able to show a direct binding. As one of the few evidences of direct interaction, Foster et al., 2013 proved that Sut1, a TF involved in filamentous growth, directly bind on Hap4 and down-regulates its expression. Soontornngun et al., 2007; Soontornngun et al., 2012 determined that Rds2 binds on Hap4 and also activates its expression. Also, Rds2 has a set of targets common with Hap4. Finally, they integrated all their data in a model explaining the interactions of transcriptional regulators involved in utilization of non-fermentable carbon sources such as *SUT1*, *HAP4*, *CAT8* and *RDS2* (Figure 2.4). The pathways regulated by the CBC and the responsible TF are summarized in Figure 2.5.

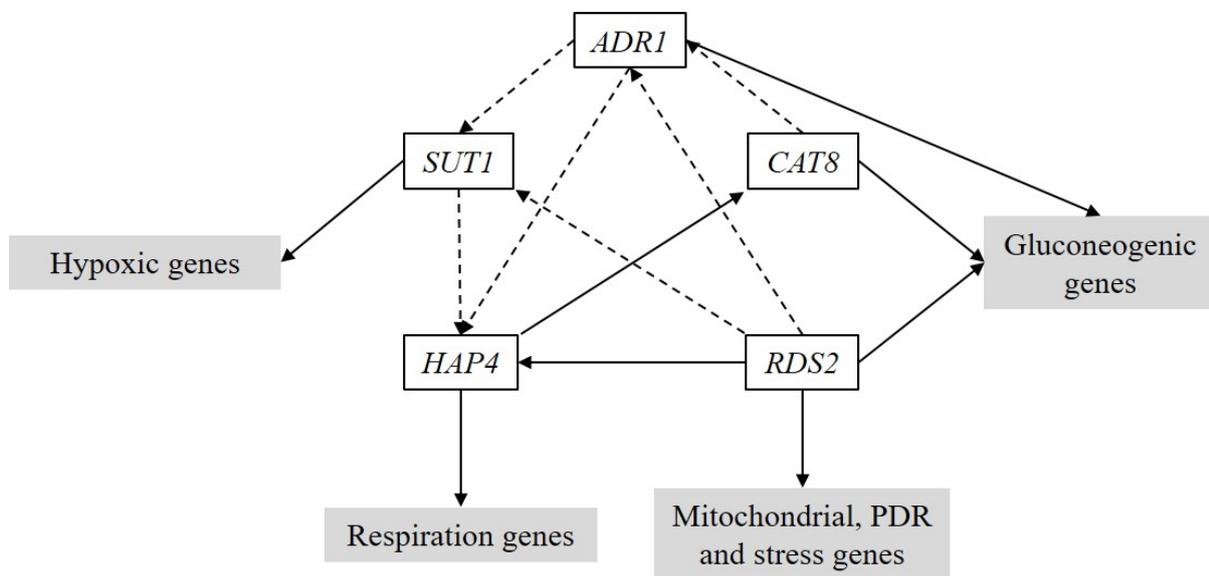


FIGURE 2.4. Interaction of transcriptional regulators involved in the utilization of non-fermentable carbon sources in *S. cerevisiae*. This figure is adapted from Soontornngun et al., 2012. Solid lines indicate binding and expression regulation. Dashed lines indicate only binding.

Hap2, Hap3 and Hap5 are conserved throughout evolution and it is quite easy to identify them in genomic sequences using homology detection. This does not hold true for Hap4 which quickly diverged. For example, even if *Kluyveromyces lactis* is quite close to *S. cerevisiae*, only two domains of less than 20 amino acids are conserved between the Hap4 proteins of each species (Bourgarel et al., 1999). In more distant species such as *S. pombe*, the activation domain of the protein is lost and only the site of interaction with Hap5 (16 aa) is conserved (McNabb et al., 1997). However, these 16 aa are really conserved across evolution and allowed to identify Hap4-like proteins in many species. Interestingly, in yeasts distant of *S. cerevisiae*, this Hap4-like proteins often have a bZip DNA-binding domain resembling the one found in Yap1. The first protein with these characteristics was called HapX and was identified

Non-fermentable carbon source

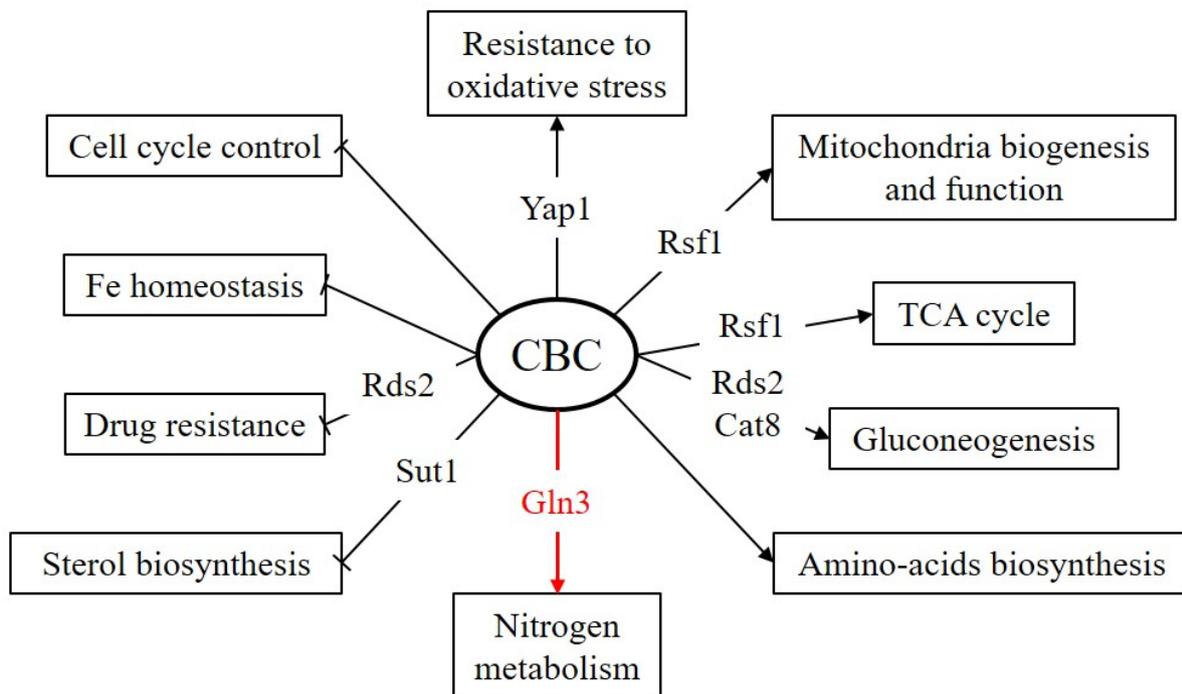


FIGURE 2.5. CBC known roles and associated transcription factors in *S. cerevisiae*. This figure sums up the roles of the CBC when cells encounter a non-fermentable carbon source. Black lines indicate an interaction between the CBC and Hap4. The red line indicates that the CBC has a role but not Hap4. An arrow indicates an activation, a perpendicular line indicates a repression. Putative supplementary TF involved are specified on the black/red lines.

in *Aspergillus nidulans* by Tanaka et al., 2002. They also showed that this protein was involved in iron homeostasis, which is an interesting feature considering the role of iron in respiration. The relationship between iron homeostasis, oxidative stress and respiration is not so surprising given that iron excess is deleterious to the cells, allowing Fenton reactions to take place and provoke oxidative stress. Conversely, iron is required in many pathways among which the synthesis of the respiratory components such as iron-sulfur clusters, for example. Additionally, other HapX proteins were found in plant pathogens (*Verticillium dahliae* (Wang et al., 2018) and *Fusarium oxysporum* (López-Berges et al., 2012)) and human pathogens *Cryptococcus neoformans* (W.H. Jung et al., 2010), *Aspergillus fumigatus* (Schrettl et al., 2010), *Blastomyces dermatitidis* (Marty et al., 2015), *Arthroderma benhamiae* (Kröber et al., 2016) and *C. albicans*, where it is called Hap43 (Hsu et al., 2011; Singh et al., 2011). In all these species, an interaction between the CBC and a HapX protein is required to mediate iron homeostasis and virulence. Even if a possible link between iron homeostasis and the CBC hasn't been explored much in *S. cerevisiae*, it would be interesting to know where *Candida glabrata* stands on this topic : is *C. glabrata*

CBC involved in respiration (given its similarity with *S. cerevisiae*) or in iron homeostasis ?

2.2.4 The iron homeostasis is linked with respiration and oxidation

Iron acquisition, besides being a key virulence factor for several fungal pathogens (Bairwa et al., 2017; Gerwien et al., 2018), is first and foremost an essential metal in a majority of organisms. This is probably because of the diversity of pathways it is involved in, such as oxygen transport, tricarboxylic acid (TCA) cycle, respiratory chains in mitochondria or Fe-S cluster biogenesis also in mitochondria (Outten et al., 2013). Furthermore, iron starvation, for example, renders cells more sensitive to oxidative stress (Blaiseau et al., 2001). Hence, regulating iron acquisition, utilization and storage is mandatory for any organism, and even more for fungi like *C. glabrata*, which encounter rapidly changing media. The yeast can face two kinds of challenge : an iron-depleted media, or an excess of iron. These challenges will be solved using different mechanisms and regulators, which are represented in Figures 2.6 and 2.7.

2.2.4.1 Response to iron-depleted media

In case of iron deficiency, *C. glabrata* combines two types of solutions to get back to normal iron levels. It can increase its iron uptake and repress iron consuming genes. All the mechanisms involved are represented on Figure 2.6.

The iron uptake is mediated through several means :

- The utilization of siderophores (I in Figure 2.6), which is one of the most efficient pathway. Siderophores are heterogeneous small molecules with a very high affinity for the iron ion (Fe^{3+}). They are usually secreted by micro-organisms (bacteria and fungi) in the extracellular media to bind the iron that is available there. One of the interest to form a complex between ferric iron and siderophores is to prevent it from undergoing Fenton reactions, thus avoiding formation of hydroxyl radicals and oxidative stress. Hence, this protects microorganisms against iron toxicity (Eisendle et al., 2006). Complexes made of iron and siderophores will then either go directly into the cell or the ions will be delivered to specific transporters sitting on the yeast surface. These transporters will then bring the iron into the cells. However, siderophore biosynthesis is impossible in *C. glabrata* because it is lacking a key enzyme, Sid1. Thus, it has to hijack xenosiderophores (siderophores synthesized by other fungi or bacteria). To that end, the yeast uses the membrane transporter Sit1 (Nevitt et al., 2011) to bring xenosiderophore-iron complexes in the cell.

- The high-affinity reductive iron pathway (II in Figure 2.6) : in *S. cerevisiae*, this pathway involves two sequential steps. The first step happens at the cell surface. It comprises the reduction of ferric iron to soluble ferrous iron by ferric reductases encoded by the *FRE* genes (*FRE1* and *FRE2*). This allows the extraction of ferric ions when they are bound to siderophores for example. The second step is the re-oxidation of the ferrous form to the ferric form by a multicopper ferroxidase (Fet3) coupled with transport into the cell by a permease (Ftr1). The last two genes are conserved in *C. glabrata* and activated during iron uptake (Srivastava et al., 2014; Gerwien et al., 2016). However, Gerwien et al., 2017 showed that even if the three putative ferric reductases they identified (including *FRE6* and *FRE8*) are induced during iron starvation and regulated by Aft1, these genes are dispensable during iron-dependent growth and don't impact extracellular iron uptake. Also, they detected no extracellular ferric reductase activity, which led them to suppose the existence of an excreted low-molecular weight non-protein ferric reductant, as in *C. albicans*. This compound would take care of the extracellular ferric reduction and compensate for the absence of surface reductases.
- The utilization of host compounds (III in Figure 2.6) : *C. glabrata* is able to use ferritin and non-protein-bound iron (FeCl₃) as iron sources in a pH-dependent manner : the lowering of the pH can increase iron solubility or provoke the release of iron from host molecules (Lestas, 1976; Kasper et al., 2014; Gerwien et al., 2018). Given that *C. glabrata* can manipulate the pH, for example in macrophages (Kasper et al., 2015), it would seem possible that it uses the mechanism during iron import. Interestingly, in *S. cerevisiae*, Diab et al., 2013 proved that the disruption of vacuolar acidification leads to lower cytosolic pH and induces expression of the Aft1/2 regulon and the cytosolic peroxiredoxin Tsa2, providing a link between iron homeostasis and pH as well as a link between iron homeostasis and redox metabolism. Also, due to contradictory reports, it is still unclear whether *C. glabrata* can use host compounds such as haemoglobin, though Srivastava et al., 2014 demonstrated that *C. glabrata* is able to grow on iron-depleted media supplemented with heme or haemoglobin. This pathway might involve Ccw14 (a cell wall protein containing the CFEM domain which shares domain homology with the *C. albicans* heme transporters Rbt5 and Rbt51), Hmx1 (a heme-binding peroxidase involved in the degradation of heme) and Mam3 (a protein with similarity to haemolysins).
- Release of vacuolar iron (IV in Figure 2.6) : vacuoles are used for iron storage in period of iron excess. When required, *C. glabrata* is able to release iron from the vacuoles thanks to the Smf3

transporter. The Fth1/Fet5 complex, composed of Fth1/Fet5 coupled to a ferric reductase, resembling the reductive uptake system of the plasma membrane (Ftr1/Fet3) could also be involved. However, once again, reports are contradictory : *FTH1* was found to be iron regulated by Gerwien et al., 2016 but deletion of *FTH1* or *FET5* did not cause sensitivity to the iron chelator BPS (Srivastava et al., 2014).

- The use of low-affinity transporters (V in Figure 2.6) : the broad-spectrum metal transporters for iron, copper and zinc can be used to import iron. In *C. glabrata* and *S. cerevisiae*, this role is probably played by the Fet4, situated at the plasma membrane (Srivastava et al., 2014; Gerwien et al., 2016).

These key genes compose the iron-regulon and are induced during iron starvation (Srivastava et al., 2015; Gerwien et al., 2016; Nagi et al., 2016). This transcriptional activation is controlled by two paralogous transcription factors, Aft1 and Aft2, which are also induced during iron starvation. In *S. cerevisiae*, their induction relies on the detection of low cytosolic iron levels by a process involving the mitochondrial iron-sulfur (Fe-S) cluster biogenesis and assembly pathway (O.S. Chen et al., 2004; Rutherford et al., 2005; Hausmann et al., 2008). Also, their regulation depends on DNA binding and nuclear localization (Yamaguchi-Iwai et al., 2002; Ueta et al., 2012; Poor et al., 2014). In *C. glabrata*, Aft1 is a strong inducer of genes involved in iron uptake at the plasma membrane and genes responsible for the cytoplasmic adaptation to low iron conditions (Srivastava et al., 2014; Srivastava et al., 2015; Gerwien et al., 2016). These last category of genes notably includes the *CTH* genes, which encode RNA binding proteins involved in the selective degradation of mRNAs encoding iron consuming proteins in *S. cerevisiae* and *C. glabrata* (see Puig et al., 2005; Puig et al., 2008 for *S. cerevisiae* *CTH1* and *CTH2* and Gerwien et al., 2016 for *C. glabrata* *CTH2*). This is actually the second mechanism used by *C. glabrata* to salvage iron during iron shortage. In *S. cerevisiae*, Aft2 shares some targets with Aft1 but is also responsible for the specific activation of intracellular iron trafficking, with genes such as the vacuolar iron transporter Smf3 and the mitochondrial iron transporter Mrs4 (Rutherford et al., 2003; Courel et al., 2005). This situation is conserved in *C. glabrata* (see Results and discussion Chapter 6). Still in the baker's yeast, Aft1 was shown to regulates its targets via the binding to the TGCACCC motif, while Aft2 binds to the ACACCC motif (Courel et al., 2005). This also seems to be conserved in *C. glabrata* (see Gerwien et al., 2016 and Srivastava et al., 2015 and Results and discussion Chapter 6). Finally, in addition to the *S. cerevisiae*-like Aft1 response under iron starvation, *C. glabrata* also possesses a *C. albicans*-like network represented by Sef1 (Gerwien et al., 2016), in which this TF regulates genes

involved in the TCA cycle, glutamate biosynthesis and iron sulfur cluster-dependent functions during iron variation.

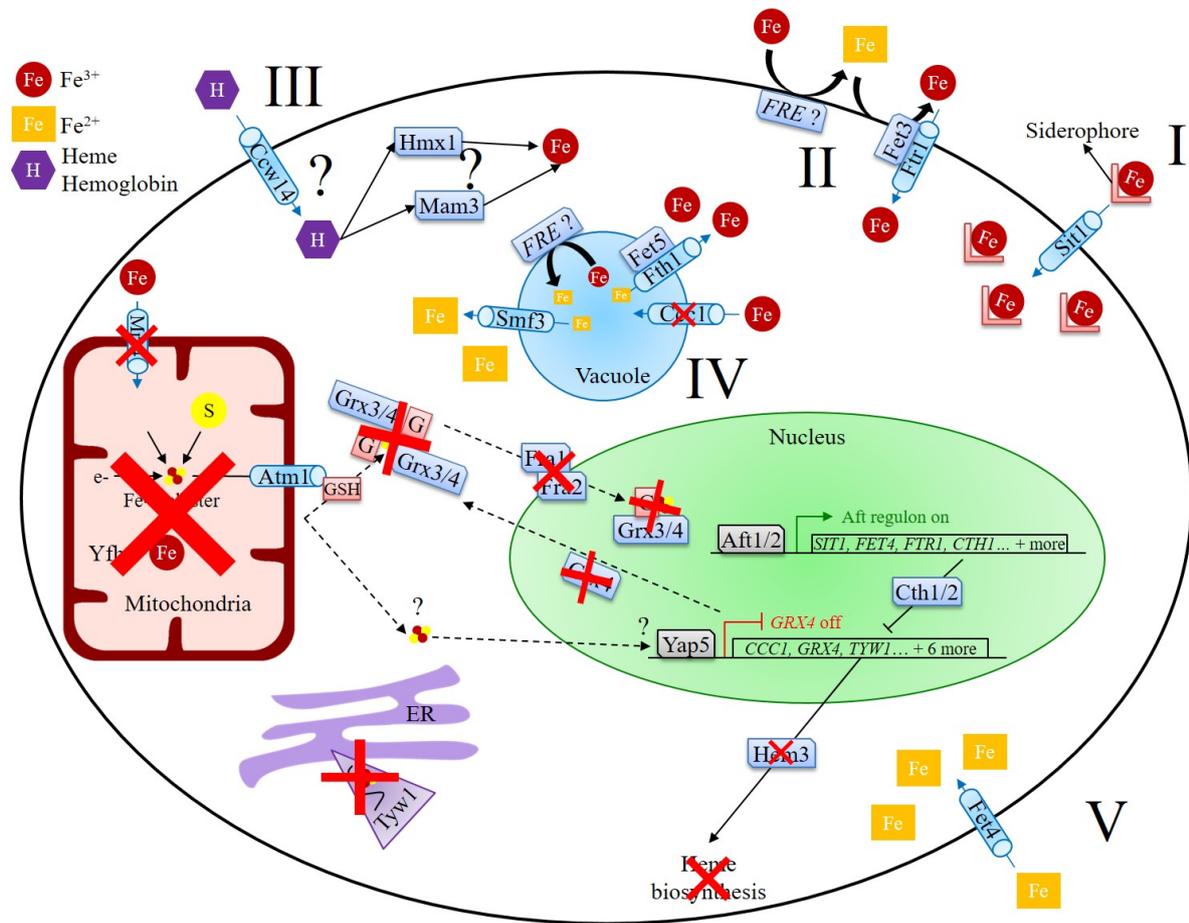


FIGURE 2.6. Mechanisms of iron-deprivation response in *C. glabrata*. This figure sums up the different mechanisms involved in response to iron starvation. Roman numeral indicates the different pathways. I is the integration of xenosiderophores. II is the high-affinity reductive iron pathway. III is the utilization of host compounds and possibly heme/hemoglobin. IV is the release of vacuolar iron. V is the use of low-affinity transporters. Red crosses indicate inactivated pathway. Dashed arrows indicate pathways proven to exist in *S. cerevisiae* and likely to exist in *C. glabrata* given the conservation of genes, targets and roles. Question marks represent unsure pathways or genes that haven't been proved to exist/to be involved yet.

2.2.4.2 Response to iron-excess

In case of iron excess, *C. glabrata* uses three types of approaches to appropriate iron levels. It can increase its iron consumption, store iron as a useful stock to deal with future iron shortage and repress iron uptake genes (Nevitt et al., 2011; Merhej et al., 2016; Sharma et al., 2016). All the mechanisms involved are represented on Figure 2.7. Overall, the iron excess response is widely conserved between *S. cerevisiae* and *C. glabrata*, because it involves the same target genes and the same TF, namely the bZIP protein Yap5 (see L. Li et al., 2008; L. Li et al., 2011; L. Li et al., 2012; Pimentel et al., 2012 for *S.*

cerevisiae and Merhej et al., 2015; Merhej et al., 2016 for *C. glabrata*). Yap5 belongs to the Yap family of TF. It is responsible for most of the aspects of the iron excess response :

- It induces the *CCC1* transporter (I in Figure 2.7), which moves cytoplasmic iron into the vacuole, thus decreasing the cytosolic concentration of iron (see O.S. Chen et al., 2000; L. Li et al., 2008; L. Li et al., 2001 for *S. cerevisiae* and Merhej et al., 2015; Merhej et al., 2016 in *C. glabrata*).
- It can also induce genes encoding for iron-sulfur cluster proteins (II in Figure 2.7) thus contributing to iron sequestration. These genes are *ISA1*, *TYW1*, *ACO1*, *RLI1*, *SDH2* and *GLT1* and play a role in mitochondria, TCA cycle, ribosome biogenesis. Additionally, Yap5 induces *HEM3*, which is important in heme biosynthesis (see Kaut et al., 2000; Noma et al., 2006; Narahari et al., 2000; Kispal et al., 2005; Dibrov et al., 1998; Filetici et al., 1996; Keng et al., 1992 for *S. cerevisiae* and Merhej et al., 2015; Merhej et al., 2016 in *C. glabrata*).
- Yap5 induces Grx4 (see L. Li et al., 2011; Pimentel et al., 2012 for *S. cerevisiae* and Merhej et al., 2015; Merhej et al., 2016 in *C. glabrata* (III in Figure 2.7), a glutaredoxin that can sense iron-sulfur clusters concentration in the cytoplasm (Mühlenhoff et al., 2010). Grx4 negatively controls the activity of Aft1 and Aft2 : in case of iron excess, there is a sufficient quantity of Fe-S clusters and they bind to Grx3 and Grx4. These complexes then interact with Aft factors, forcing them to leave their promoter targets (Ueta et al., 2012) and stopping the induction of iron uptake genes. Interestingly, Yap5 expression is induced by the same Fe-S clusters that are produced following Yap5 induction (L. Li et al., 2012). Miao et al., 2009 demonstrated that Atm1, a mitochondrial inner membrane transporter, is essential to the excretion of Fe-S clusters outside of the mitochondria and thus to the regulation of Aft1 and Aft2. It was also shown that GSH is essential to this regulation because GSH depletion causes constitutive activation of the Aft1/2 iron regulon (Rutherford et al., 2005). The requirement for GSH might originate from its role as a Fe-S cluster ligand within Grx3/4 complexes. Furthermore, GSH role in OSR is secondary to its essential role in iron metabolism (Kumar et al., 2011).

Besides, Srivastava et al., 2014 established the involvement of *YFH1* in iron excess. Yfh1 is a mitochondrial matrix iron chaperone which oxidizes and stores iron. This protein is required for Fe-S clusters biosynthesis in the mitochondria, and its deletion leads to constitutive activation of the high-affinity iron uptake pathway as well as an increase in the cellular iron content. Sharma et al., 2016 also found that upon iron excess, Ftr1 membrane permeases move to the vacuole in a Vps34 kinase-dependent

manner (IV in Figure 2.7). This prevents cells from importing iron through the pathway mentioned earlier (the high-affinity reductive iron pathway). However, we don't know exactly how these two systems work and further researches are required. Other features of the iron excess response of *C. glabrata* will be detailed in (Results and discussion Chapter 5).

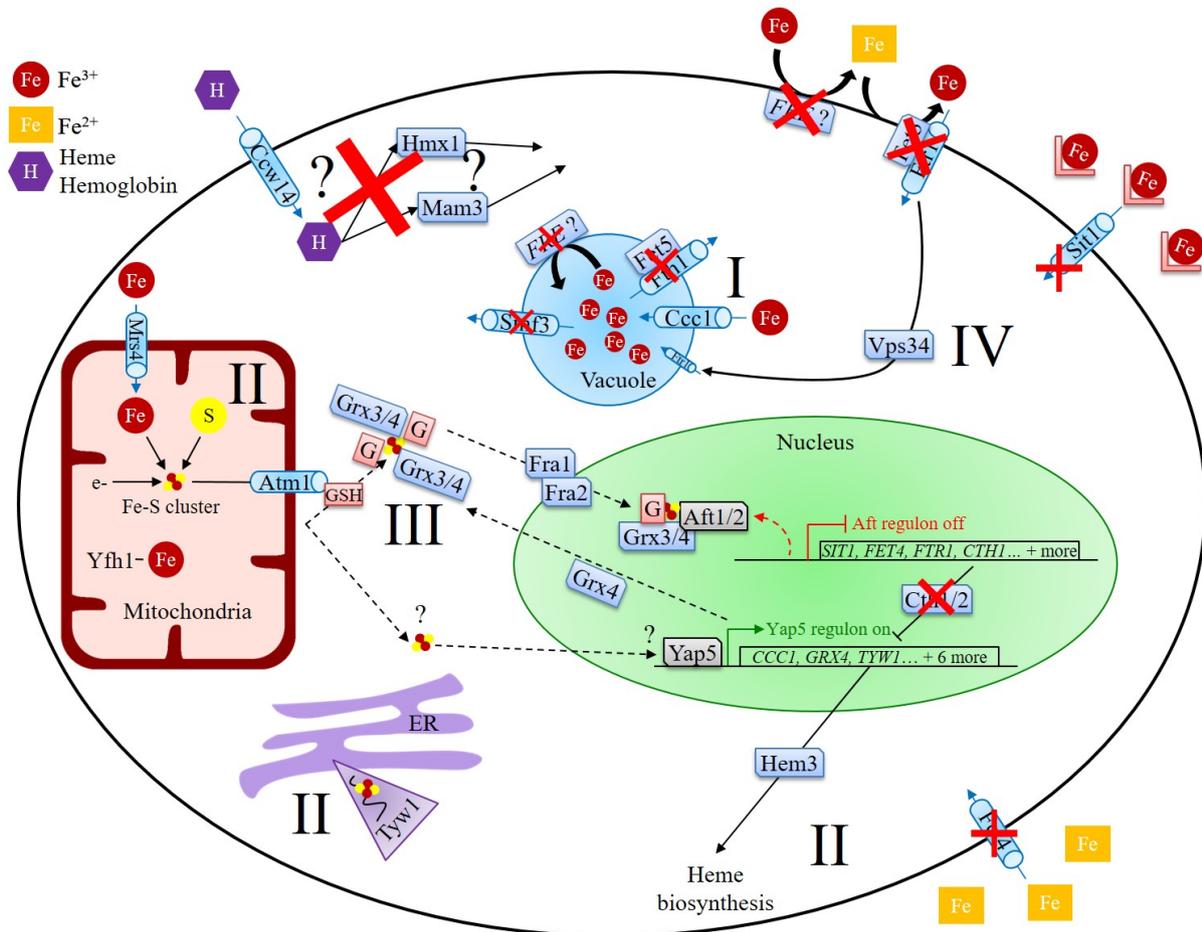


FIGURE 2.7. Mechanisms of iron-excess response in *C. glabrata*. This figure sums up the different mechanisms involved in response to iron excess. Roman numeral indicates the different pathways. I is the induction of the *CCC1* transporter. II is the induction of iron consuming genes. III is the induction of the Grx mediated inactivation of Aft factors. IV is *Ftr1* relocation to vacuole in a *Vps34*-dependent manner. Red crosses indicate inactivated pathway. Dashed arrows indicate pathways proven to exist in *S. cerevisiae* and likely to exist in *C. glabrata* given the conservation of genes, targets and roles. Question marks represent unsure pathways or genes that haven't been proved to exist/to be involved yet.

Candihub, the study of transcriptional networks of *Candida glabrata* stress responses

Over the past few years, incredible technical improvements were made, such as Next-Generation Sequencing or RNA-seq. This type of experiments now generates huge amounts of data that need to be carefully analyzed in order to answer the biological questions we ask. The methods to analyze such data improved in parallel of the technical evolution. One major consequence of these technical and analytical enhancements is the change of paradigm towards the things we study : whereas before we were limited to single sided investigations of simple phenomena, we are now able to integrate several types of data (for example, transcriptomic data, proteomic data, microscopy. . .) to build models precisely describing and explaining very complex events. This allows to study events involving a lot of players interacting together, for instance genes expression during an organism stress response. This global approach at a higher level is called systems biology. F. Horn et al., 2012 describe it as “a comprehensive quantitative analysis of the interactions of a large number of functionally diverse and frequently multifunctional sets of elements, such as genes, proteins, metabolites, which produce coherent behaviours in time and space.”.

3.1 Networks : a model to represent connections between various elements

One of the goals of systems biology is to unravel the interactions and the links existing between elements of one or several processes. This leads to the creation of networks, defined by Wasserman et al., 1994 as “systems whose elements are somehow connected”. Basically, a network represents which elements are connected or interacting, or not. An example of network can be found in Figure 3.1. Nodes (the circles on Figure 3.1) and edges (the lines between circles on Figure 3.1) are the basic components

of a network. Nodes represent units in the network (genes, proteins, metabolites, neurons...), while edges represent the interactions between the units (gene A is induced by gene B, protein A and B interact together in a complex, metabolite A is a precursor of metabolite B, neuron A sends information to neuron B...). Edges can be weighted to represent more accurately a phenomenon. For instance, if a metabolite can be the precursor of two metabolites but the chemical environment favours one type of reaction, the edge representing the favoured reaction will have more weight.

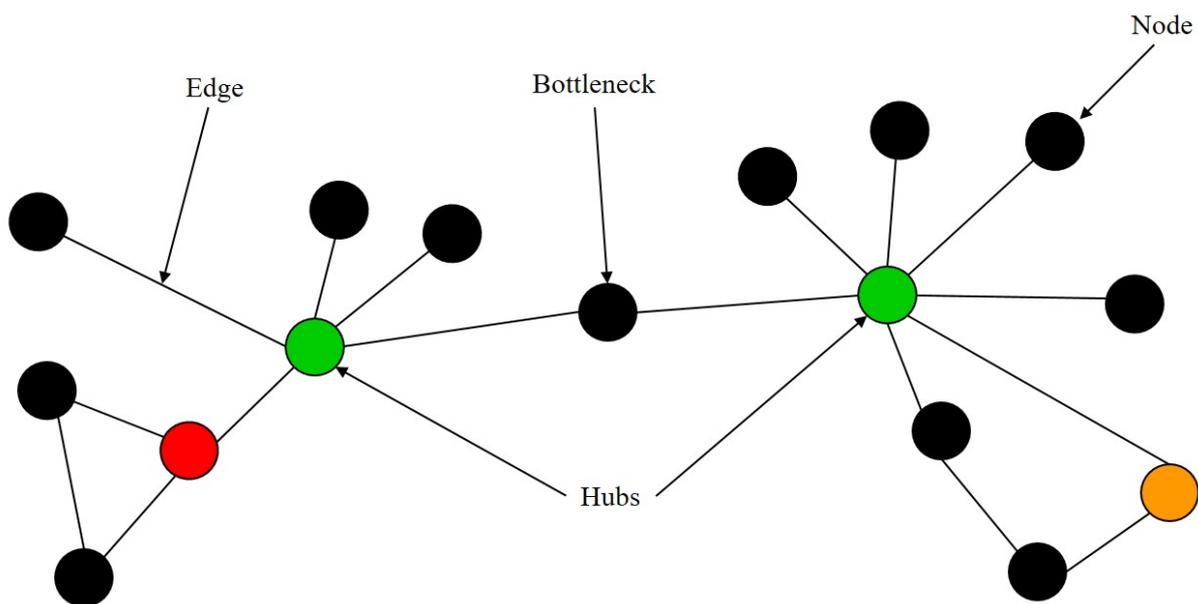


FIGURE 3.1. Example of a network and some of its characteristics. This network has 16 nodes and 17 edges. Red node has a degree of 3. Orange node has a degree of 2. Green nodes have the highest values of betweenness.

Hence, the structure (or the topology) of a network in itself is a gold mine of information. This information is extracted from a network by calculating numerous indices and index : distances, density, costs... Among the most important properties of a network are found degree and betweenness centrality. Degree (or connectivity) is the number of edges that connect a node. In Figure 3.1, red node has a degree of 3 while orange node has a degree of 2. Interestingly, the calculation of the degree allows to define some nodes as hubs, which are strongly connected to the rest of the network (green nodes in Figure 3.1). Betweenness of a node is the number of shortest paths between two other nodes that pass through the studied node. In other words, it measures the centrality of a node in a network. Nodes with high betweenness essentially serve as connections between different portions of the network (*i.e.* interactions must pass through this node to reach other portions of the network). Still in Figure 3.1, the nodes with highest betweenness are the nodes in green. This measure is linked with the notion of bottleneck : a bottleneck (see Figure 3.1) is a node with a high betweenness, which removal would split the network in 2

or more smaller subnetworks. All these measures help to characterize the networks and draw conclusions from them.

3.2 The transcriptional regulatory networks

Networks studies can be applied to several fields, amongst which transcriptional regulation of the genes in a cell or an organism. Transcriptional regulatory networks play an important role in biological processes, because they define groups of co-regulated genes that act together to fulfill specific functions at the proper time and place. For example, it is particularly useful to determine the involvement of genes during stress response or to define the components of a pathway. Transcriptional regulation is the means by which cells regulate the genes expression. It is mediated by two things : cis-regulatory elements and transcription factors. Cis-regulatory elements are regions of non-coding DNA often found in promoters which regulate the transcription of neighboring genes thanks to the binding of Transcription Factors (sequence specific DNA binding proteins that regulate a sets of target genes), hence the existence of interactions. The fact that the transcriptional relationship between genes and TF is not reciprocal (a TF binds on a gene promoter to modify its expression, not the other way around) introduces new elements in the networks :

- Nodes will have different roles, thus all nodes won't be equivalent. There will be two categories : target genes bound by TF and the TF themselves.
- Edges will have a specific direction : an edge will always leave a TF to reach a target gene.
- Given that a TF can activate or repress a target gene, not all edges will be equivalent.

These elements have to be taken into account to obtain proper Gene Regulatory Networks (GRN).

Babu et al., 2004 showed that global transcriptional regulatory networks have scale-free topologies, which means that they are heterogeneous, with the majority of the regulators having relatively few target genes, and a few regulators having a large number of targets. They also demonstrated the existence of genes with a lot of inbound connections, *i.e.* hubs. These are especially interesting because they are central in the network and are involved in a lot of processes. For this reason, hubs usually are essential genes with high conservation and key roles in organizing a coherent cellular functioning. In case of pathogens or cancerous cells, they often are promising drug targets (Jonsson et al., 2006). Noteworthy, a hub can also be a TF bound by several other transcription factors. This implies a hierarchical organization

in the TF, with high level master regulators (Msn2 and Msn4, for example) and low level regulators (Sef1 in *C. glabrata*, for instance). Obviously, the higher the TF is in the hierarchy, the more crucial it is for cell functioning and survival. Finally, some TF are useful in a lot of different conditions, which make them hubs because of their numerous co-regulatory associations with various other TF (Balaji et al., 2006). This could be the case of Yap1, for example. Thus, precisely identify hubs could have an impact on medical outcome as well as understanding whole pathways in an organism.

3.3 The Candihub project : deciphering stress responses in *Candida* species using transcriptional regulatory networks

As explained in the previous parts (Introduction Chapters 1 and 2), the ability of *Candida glabrata* and *Candida albicans* to adapt and resist to variations of their environment is a key parameter to acquire virulence features and be successful pathogens. As pathogens, they are confronted with a range of niches, each one with a varying pH, temperature, redox potential, carbon source or metal availability. They are also confronted with violent attacks from the immune system. All these brutal changes in the yeast environment cause stresses that need to be addressed in order for *Candida* to survive and thrive. These stresses trigger several pathways that can be general stress responses or specialized responses adapted to the very nature of the stress. The stress response pathways usually include a lot of players and several layers of regulation, which render the pathways hard to decipher. This difficulty is accentuated by the multitude of interactions between the players of the pathways, creating huge maps of interactions. As such, these phenomena would be best studied and understood using a transcriptional regulatory networks approach.

However, performing such powerful systemic studies requires a lot of data to precisely describe the events to investigate. Unfortunately, there were only a few available high-throughput data in *Candida* species, especially compared to *Saccharomyces cerevisiae* as shown in Table 3.1, even though *C. glabrata* and *C. albicans* genomes have been available since 2004 (Dujon et al., 2004; Jones et al., 2004). Up to 2013, the Gene Expression Omnibus from the NCBI contained almost 1400 *S. cerevisiae* high-throughput datasets (including expression profiling and genome binding/occupancy profiling), while it displayed only 134 and 30 *C. albicans* and *C. glabrata* high-throughput datasets, respectively. Hence, *Candida* species were clearly lagging behind *S. cerevisiae*. As a consequence, in 2013, several of the interactions networks of *S. cerevisiae* were deciphered, while most of *Candida* networks were unknown.

Species	Transcriptomic datasets	Genomic datasets
<i>Saccharomyces cerevisiae</i>	1091	306
<i>Candida albicans</i>	110	24
<i>Candida glabrata</i>	26	4

TABLE 3.1. Number of high-throughput experiments performed in *S. cerevisiae*, *C. albicans* and *C. glabrata* up to 2013 included. Transcriptomic datasets include expression profiling by array, expression profiling by genome tiling array and expression profiling by high-throughput sequencing. Genomic datasets include genome binding/occupancy profiling by array, genome binding/occupancy profiling by genome tiling array and genome binding/occupancy profiling by high-throughput sequencing.

3.3.1 Description and goals of the Candihub project

The previous assessments and the will to understand *Candida* stress responses and virulence led to the implementation of the Candihub project in 2014 for the duration of 5 years. The Candihub project, which is funded by the french Agence Nationale de la Recherche, includes three partners :

- Christophe d'Enfert and his team, based at the Institut Pasteur in Paris. He was part of the international consortium that annotated the *C. albicans* genome and he created the first public database for accessing genomic information in *C. albicans* (d'Enfert et al., 2005). He participated to the first transcriptomic analyses conducted in this species and also discovered and characterized several new transcriptional regulators involved in morphogenetic switching and biofilm formation (Bonhomme et al., 2011; Chauvel et al., 2012), which are key parameters of virulence. His team was also involved in the construction of tools for large-scale genetic screening in *Candida* species, such as a collection of *C. albicans* strains overexpressing genes of signal transduction pathway (Chauvel et al., 2012) and a collection of *C. glabrata* strains deleted for more than 600 individual genes, including many TFs (Schwarzmueller et al., 2014).
- Frédéric Devaux and his team, based at the Institut de Biologie Paris-Seine and Gaëlle Lelandais from the Université Paris-Saclay. They have developed experimental and *in silico* strategies to characterize the structure of transcriptional regulatory networks in the model yeast *S. cerevisiae*, in which they described the networks of TFs involved in several environmental stress responses (Palková et al., 2002; Fardeau et al., 2007; Salin et al., 2008). They also participated in the creation of the first public repository for transcriptome data (Lelandais et al., 2004). They performed analyses in other yeast species, including *C. albicans* and *C. glabrata*, in order to analyze the evolution of the transcriptional regulatory networks (Banerjee et al., 2008; Goudot et al., 2011). Additionally, they conducted some of the first transcriptome analyses in *C. glabrata* (Lelandais et al., 2008)

and published the first RNA sequencing study of multidrug resistance in *C. albicans* (Dhamgaye et al., 2012).

- Jean-Michel Camadro, at the Institut Jacques-Monod. He created the Proteomics core facility of the IJM and has a strong expertise in mass spectrometry applied to structural biology (Combes et al., 2009) and protein/protein interactions (Komarova et al., 2011). His team developed protocols in quantitative proteomics that allow the accurate quantification of more than 3.500 expressed proteins in simple eukaryotes such as *C. glabrata* and *C. albicans* (Léger et al., 2016). Jean-Michel Camadro also collaborated with bioinformaticians and proposed mathematical models of metabolic adaptation of yeast to oxygen in relation to iron homeostasis and oxidative stress (Achcar et al., 2011).

The creation of the Candihub project fully benefited from the development of new resources for *Candida* species, such as deletion libraries. Noble et al., 2010 and Schwarzmüller et al., 2014 created collections of strains possessing one deleted gene for *C. albicans* and *C. glabrata* respectively. These libraries account for 11% and 12% of their respective genomes. Such deleted strains can be useful, for example, to measure the transcriptomic impact of a transcription factor, for example. Candihub also benefited from the development of genome editing techniques such as CRISPR (Vyas et al., 2015), because C. D'Enfert's team used it to engineer new strains of *C. albicans*.

Candihub aims to decipher the *C. glabrata* and *C. albicans* transcriptional regulatory networks involved in the adaptation of the two species to changing environments using data produced with genomic and transcriptomic experiments (see Introduction Part 3.3.2). The global analysis of the topology and functioning of the interaction networks that connect the genes of *Candida* species is an essential step towards the understanding of their coping mechanisms as well as the functioning of the whole organisms. The topologies of these networks might also lead to the identification of regulatory hubs (key genes or master regulator TF), which may very well be relevant targets for antifungal therapy, as shown in Altwasser et al., 2012 : in this paper, the authors gathered information available on *C. albicans*, such as expression profiles and known interactions, to infer genome-wide gene regulatory interaction networks. Afterwards, they identified regulatory hubs in the network, 15% of which are already described in literature as influenced by antifungal drugs. This shows the potential of networks studies.

3.3.2 Building the Candihub networks

This leaves open key questions : how can such networks be built ? Which experiments can be performed ? On which strains ? These questions will be addressed in the next parts.

3.3.2.1 Types of *C. glabrata* strains used in this work

This part will briefly explain the different strains used in the *C. glabrata* part of the Candihub project.

3.3.2.1.1 Δ HTL strain

During my PhD, I worked with the Δ HTL strain that was constructed by Jacobsen et al., 2010. This strain is derived from the ATCC2001 background (also called CBS138 background). It was obtained by deleting the *HIS3*, *TRP1* and *LEU2* genes in the ATCC2001 strain using the dominant nourseothricin resistance marker *SATI* (Reuss et al., 2004; J. Shen et al., 2005) and the associated *SATI* flipper method. Briefly, they amplified homologous regions of 500 bp flanking a gene of interest and ligated them into a plasmid already containing a *SATI* marker under a strong promoter, a recombinase and a *SATI-caFLP* gene. This gene encodes a site-specific recombinase *FLP*, under control of an inducible promoter. There are also direct repeats of the *FLP* recombination target sequence (FRT) encapsulating the cassette (Fig 3.2, panel A). They repeated this operation for the three genes of interest and created gene deletion cassettes for *HIS3*, *TRP1* and *LEU2*. The deletion cassettes were then transformed by electroporation into the ATCC2001 strain, as described in Köhler et al., 1997; Reuss et al., 2004 for example. Afterwards, they plated the transformants on a media containing nourseothricin, which allowed the identification of cells who had recombined with the plasmid containing the resistance *SATI* gene (Fig 3.2, panel B). The next step consisted in activating the inducible promoter of the recombinase, thus causing the excision of the *SATI* cassette and rendering cells sensitive to nourseothricin back again (Wirsching et al., 2000). This whole deletion thus leaves only a FRT site on the genome (Fig 3.2, panel C). Additionally, they tested the excision of the cassettes by measuring nourseothricin sensitivity of the transformants, and checked them by PCR and southern blot to account for correct genomic integration (Fig 3.2, panel C). The Δ HTL was the origin strain of all modified strains (gene-deleted or gene-tagged) that I used in the chapters detailing my results part and mainly played the crucial role of “control strain”.

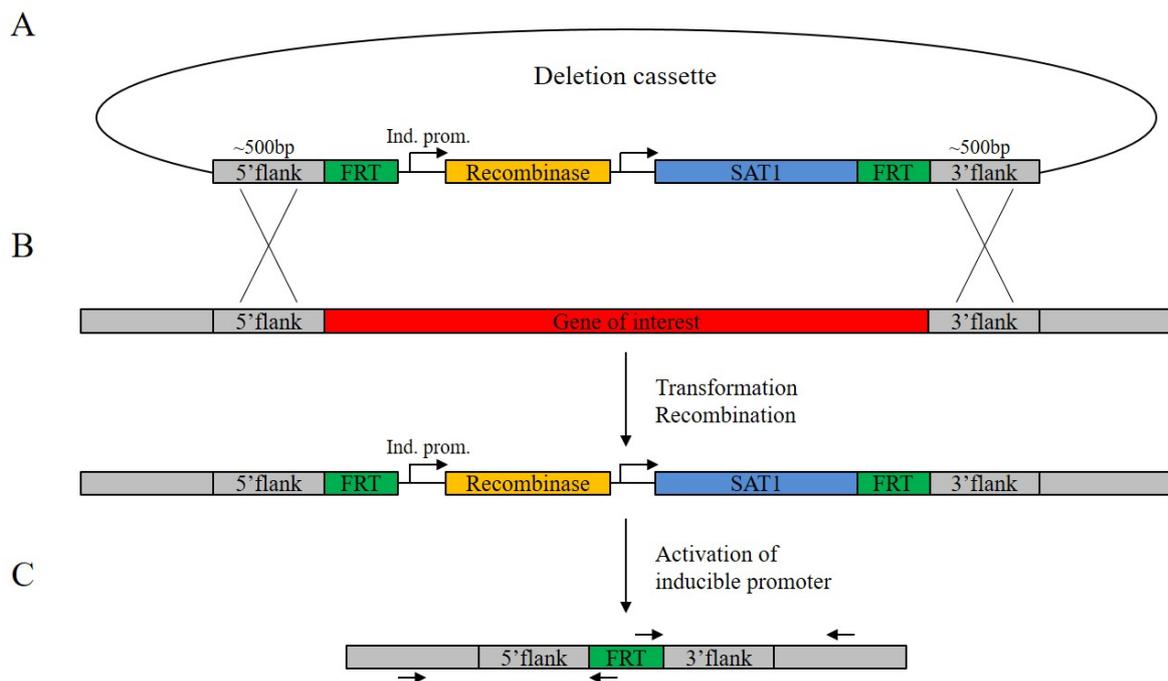


FIGURE 3.2. Construction of ΔHTL strain. The 5' and 3' flanks are adapted to the flanking regions of the gene of interest (*HIS3*, *TRP1* or *LEU2*). Arrows indicate the PCR verifications. Adapted from Jacobsen et al., 2010.

3.3.2.1.2 TF deleted strains

Several experiments required strains deleted for a transcription factor, such as transcriptomic experiments designed to determine the impact of a TF on gene expression. These deleted strains were obtained from pre-existing collections (Merhej et al., 2015; Schwarzmüller et al., 2014), all coming from the parental ΔHTL strain. Most of the strains came from the Schwarzmüller collection (Schwarzmüller et al., 2014). They took an approach of targeted gene disruption with homologous flanking regions. They used the fusion PCR technique (Noble et al., 2005) to build deletion cassettes containing 500 bp homologous flanking regions and the *NATI* marker. They also added unique barcode identifiers in the deletion cassettes. After electroporation and transformation, they selected the recombinants on nourseothricin and tested the correct genomic integration by PCR and southern blot as well as the absence of the target genes, to avoid the presence of a duplication of the target gene elsewhere in the genome.

3.3.2.1.3 myc-tagged TF strains

Chromatine Immuno-Precipitation experiments required strains with a tagged for transcription factor. We chose the myc epitope (Evan et al., 1985; Munro et al., 1987). myc-tagging cassette comes from the M. Longtine's plasmids (Longtine et al., 1998) collection. It contains 13 myc epitopes and

an auxotrophic marker. The cassette was amplified by PCR with oligonucleotides containing homology sequences flanking the desired genomic insertion points in 5'. After purification of the PCR products, this DNA was transformed into the cells using a standard yeast transformation protocol (Schiestl et al., 1989) and we plated these cells on selective media. In this clones, we verified that the myc-insertions were in phase with the target genes using PCR and Sanger sequencing (Sanger et al., 1977). We also checked the tagged protein size and production by western blot (Merhej et al., 2015).

3.3.2.2 Approaches to build networks

There are two options to build a network : either a combination of experimental data and mathematical modelling or a fully experimental network determination.

3.3.2.2.1 Mathematical inference of regulatory networks from experimental data

Network inference is a systems biology approach that predicts regulatory interactions between genes based on transcriptomic data such as RNA-seq or microarrays. Basically, the structure of a Gene Regulatory Networks is reconstructed from expression data of genes responding to a stimulus, like a stress. It presumes the existence of such data, otherwise, the network inference can't be performed and the GRN can't be reconstructed.

The principle is to use these expression data as an input to model the network. Lots of mathematical modelling can be used, each with its own pros and cons, but the choice of the method will be mainly guided by the biological question, the size of the network and the amount of available data. For example, time-consuming methods won't be used to reconstruct a genome-wide network : this fact is related to the curse of dimensionality, which states that the number of possible network structures increases exponentially with the number of genes (Schulze et al., 2016) (in some cases, this can be bypassed by the prediction of the smallest number of interactions needed to fit the measured data). Or small datasets cannot be used to reconstruct genome-wide networks, hence the need to carefully choose the model.

If the network to reverse engineer is large, it is of interest to use algorithms that are computationally fast and use simplified mathematical models. These models can be based on correlation between genes predicting undirected edges (for example, correlation-based algorithms (Stuart et al., 2003)) or causal relations between genes using weighted, directed edges (regression-based algorithms (Hecker et al., 2009)). The resulting networks allow to study the topology, essentially thanks to the calculation of node-degree distribution and betweenness centrality. With these indices, it is easy to identify hubs or

genes with a large influence on the network. If the desired network is smaller, one can use more sophisticated algorithms taking into account supplementary biological aspects, such as the presence of known transcription factor binding sites in promoters. These algorithms are usually based on Boolean logic (Müssel et al., 2010), Bayesian networks (Hartemink et al., 2001), differential equation systems (Vlaic et al., 2012) and linear (Weber et al., 2013) or nonlinear differential equation systems (X. Yang et al., 2012). The smaller the network, the easier it is to have solid results (given the data provided are good). The usual workflow of GRN inference can be found in Figure 3.3. Additional information can be found in Linde et al., 2015; Dix et al., 2016; Guthke et al., 2016; Schulze et al., 2016.

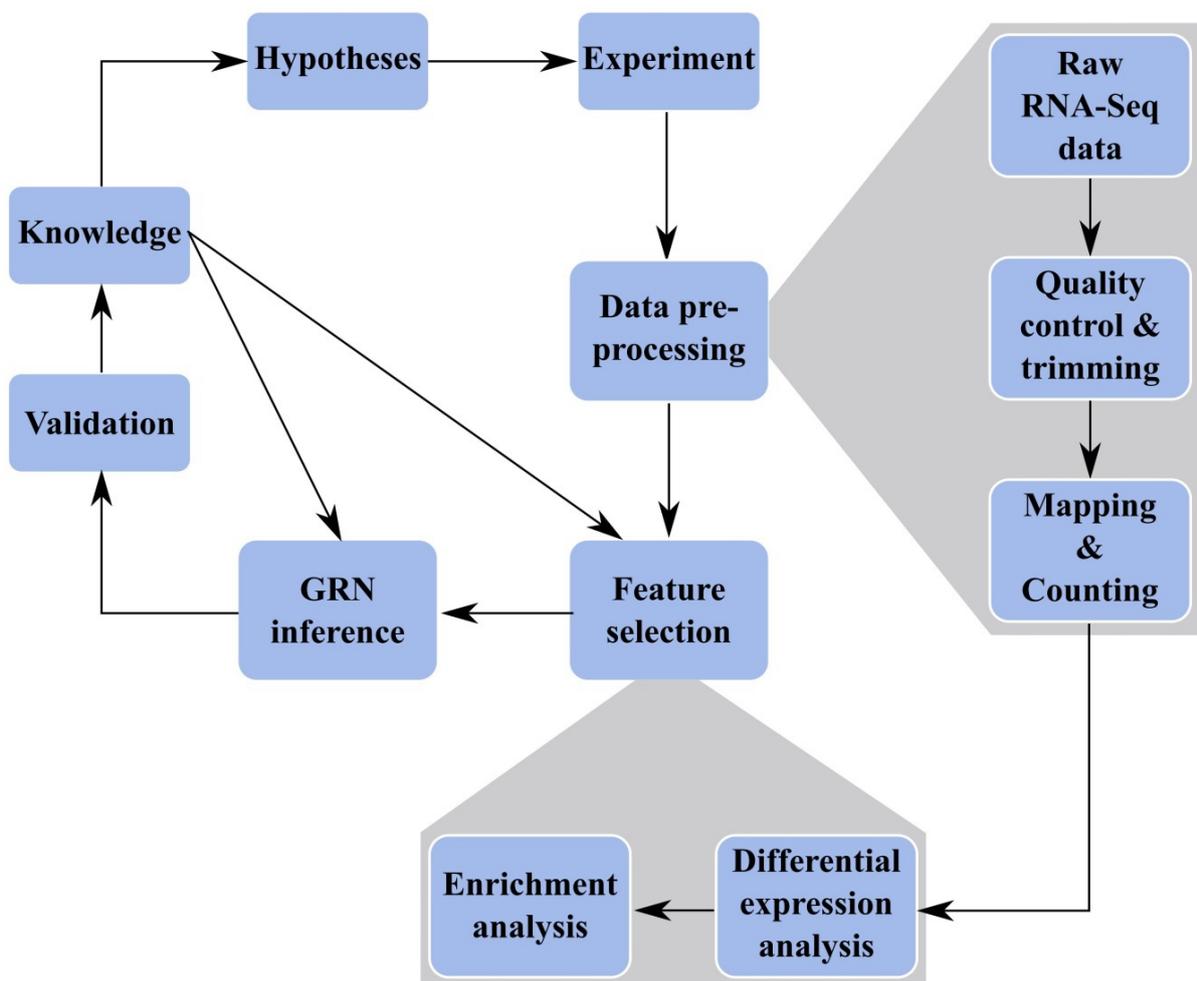


FIGURE 3.3. Workflow of GRN inference. This figure is from Linde et al., 2015. It represents the exchanges between experiment and computational processings. Experiments produces transcriptomic data that will be processed. Feature selection and modelling will allow to infer a GRN. This GRN is then confronted to experimental data for validation or adaptation of the model.

3.3.2.2 Direct determination of regulatory networks

The other way to build a gene regulatory network is to use only experimental data. It is likely to be more time-consuming and more expensive than network inference, but the results are usually more reliable (for example, Margolin et al., 2007 showed how small variations can create fake positive interactions in a reconstructed network). Besides, it is also a solid base for future network inferences. Functional genomics has given powerful approaches to efficiently describe the set of targets associated to a specific TF. For instance, genome-wide Chromatin Immuno-Precipitation (ChIP) experiments can determine a complete list of the promoters bound by a TF. This can be coupled with transcriptome analyses of loss or gain of function TF mutants that identify genes which expression depends on a TF. The association of both types of experiments proved fruitful several times, as almost all transcriptional regulatory networks of *S. cerevisiae* have been described (Harbison et al., 2004). The techniques chosen for the Candihub project will be explained in the following parts.

Analyses of the impact of TF mutations on expression by microarrays Microarrays have been used for a long time to study gene expression (P.O. Brown et al., 1999): researchers began to use them in the middle of the 90's. Since then, this technique has been well perfected, especially the manufacturing of the chips wearing the oligonucleotides probes and the amount of probes available on a single chip, but the idea remained the same : to study transcriptomics at a genome-wide level in a single experiment. Microarrays are now opposed to the much more recent RNA-seq technique, which offers new interesting features such as ability to detect novel or rare transcripts, wider range of expression quantification and higher specificity and sensitivity. However, despite the prices drop of RNA-seq, a lot of labs keep using microarrays, mainly because they are faster, cheaper, easier to use and provide a simple way to explore transcriptomes in a systematic manner. Also, RNA-seq is far from being exempt of biological and statistical biases and we have much more perspective and expertise on microarray biases because we have been experimenting with them for a longer time span. For a summary of biological biases encountered with microarrays, see Jaksik et al., 2015, who listed the factors affecting microarray reliability.

The power and universality of DNA microarrays is based on the specificity and affinity of complementary base-pairing. Briefly, arrays of thousands of DNA probes are printed on glass microscope slides using a robot. The probes are short sequences designed to match parts of the sequence of known or predicted open reading frames. To compare the relative abundance of each of these gene sequences in two RNA samples (for example, the total mRNA isolated from two different cell populations, such as

a control population and a population deleted or overexpressed for a TF), the two RNA samples are first extracted from the cells and reverse-transcribed into cDNA. Then, the cDNA samples are labelled using different fluorescent dyes. Most of the time, the dyes are Cy3 and Cy5. They are then mixed and hybridized with the probes on the microarray (Figure 3.4). For a review on biases introduced by hybridization and the possible corrections, see Koltai et al., 2008.

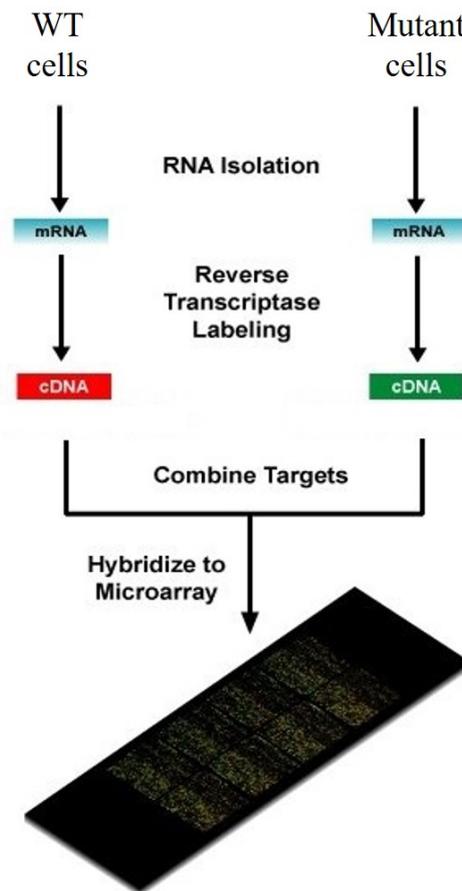


FIGURE 3.4. Microarray experiment workflow. This figure represents the different steps required in a microarray experiment.

After hybridization, the microarray is read by a scanner that illuminates each spot and measures fluorescence for each dye separately. These fluorescent measurements are used to determine the ratio, and in turn the relative abundance of the sequence of each specific gene in the two mRNA samples. A pre-processing step is required here, as they are biases caused by the scanner, impact of background, etc... It can also result of the chemical properties of the fluorescent dyes used : for instance, Cy5 is more sensitive to ozone than Cy3, which means that the experimental environment can cause the faster degradation of Cy5. All these biases are solved using pixel averaging, background subtraction, transcript

intensity summarization and removal of non-informative/not expressed transcripts (Cordero et al., 2007; Welle, 2013).

Afterwards, bio-informatics treatment will merge the fluorescence of spots corresponding to the same gene and apply a normalization, generally performed using LOESS method (Cleveland, 1979; Cleveland et al., 1988; Cleveland, 1981; M.C. Yang et al., 2001). Other normalization methods are reviewed in Do et al., 2006. Finally, expression data are put through statistical tests (linear tests such as t-tests, or Bayesian models, see Quackenbush, 2006 for review) to check for genes or group genes which expression statistically differs between the two tested conditions. Once vetted, these results can be used to perform prediction analyses, or clustering and gene enrichment analyses. This may lead to new functional annotations for unknown genes as well as network inference of regulatory interactions (see previous part) ; microarrays are useful to measure the impact of a TF deletion or overexpression and allows to identify direct and indirect target genes of a TF.

Analyses of TF binding sites by ChIP-seq One of the major flaws of transcriptomic studies is that they don't permit the identification of sole direct targets. Genes which expression varies upon a transcription factor mutation can be directly bound by a TF or can be downstream in the same pathway : in both case, gene expression will change but that does not always imply that the gene is a target of the transcription factor. On the opposite, if a gene expression doesn't change upon a TF deletion, it might be because this gene is not the target of the studied TF or because several TFs have a redundant role in regulating this gene. An efficient way to solve these problems is to study the binding location of the TF in the genome. One of the best course to achieve that is to perform Chromatin Immuno-Precipitation (ChIP) experiments followed by next-generation sequencing.

ChIP is a valuable method to investigate protein-DNA interactions. Since its discovery it has been indispensable to identify binding sites and patterns of DNA-interacting proteins, such as transcription factors or histones. The first ChIP experiment was performed in the early 80's (Gilmour et al., 1984; Gilmour et al., 1985) and was designed to study precise locations of the genome. ChIP became a routine after 2000 and in 2007, three labs (Barski et al., 2007; Johnson et al., 2007; Mikkelsen et al., 2007) published the first ChIP-seq experiments, producing genome-wide analyses of transcription factor binding sites. This was a game changer, especially considering the decreasing cost of sequencing nowadays. Additionally, a lot of ChIP derivatives were developed. Most of them are reviewed in Collas, 2010.

Even if the endpoint techniques of ChIP have changed, the core protocol hasn't changed much (Figure 3.5). It starts with formaldehyde fixation of the biological material of interest. Formaldehyde is a very reactive agent that interacts, through its nucleophilic core, with amino groups of amino-acids and side chains of DNA bases. Fixation preserves the DNA-proteins associations that would otherwise be lost or disturbed during the next steps. The cross-linking reaction is stopped by adding glycine. The cells are then lysed. This step removes cytoplasmic and membrane proteins, breaks the nucleus and help removing the non- cross-linked proteins. After lysis, a suspension of soluble chromatin is obtained by sonication : the chromatin is sheared to $\simeq 300\text{-}500\text{bp}$ fragments. This step is important, because the shorter the DNA fragments are, the higher the resolution of the final results is. However, there is a minimum size determined by the endpoint analysis : for instance, fragments should not be shorter than sequencing reads in case of ChIP-seq. Chromatin can also be sheared using specific DNA endonucleases, such as micrococcal nuclease, or a combination of sonication and nuclease treatment. The chromatin is then subjected to immunoprecipitation with an antibody against the protein of interest. The specificity of the antibody will determine the quality and reliability of the results : antibodies should have a high affinity for the antigen under stringent conditions, and should not cross-react with other proteins. In any case, a negative control precipitations in the absence of antibody (this is called the input) should be performed. After the IP, the crosslinks are reversed and the immuno-precipitated DNA fragments are then purified and analyzed (see below for the different analyzes, but the idea behind ChIP is that if the protein under investigation is associated with a specific genomic region, DNA fragments from this region should be enriched in the immuno-precipitate).

This type of protocol raises a few remarks :

- The formaldehyde cross-linking step is quite empirical because little is known about its specificity and efficiency, and variability in cross-linking from one experiment to the next have been reported. Still, if the crosslinking time is too short, the IP will fail. If it's too long, it will prevent chromatin shearing during sonication. Thus, this step must be adapted to the cells, the proteins, the organism... For some proteins, cross-linking turned out to be difficult or even impossible (Gavrilov et al., 2015), and it can also cause changes to the chromatin composition, thus decreasing the ChIP efficiency (Beneke et al., 2012). Formaldehyde might also be the reason for non-specific enrichments of highly expressed genes (Park et al., 2013; Teytelman et al., 2013), a well-known fact among labs performing ChIP. Besides, it is a possible carcinogen. The next obvious question is "Can we use something else ?". The answer is yes : several chemical compounds (acridine orange,

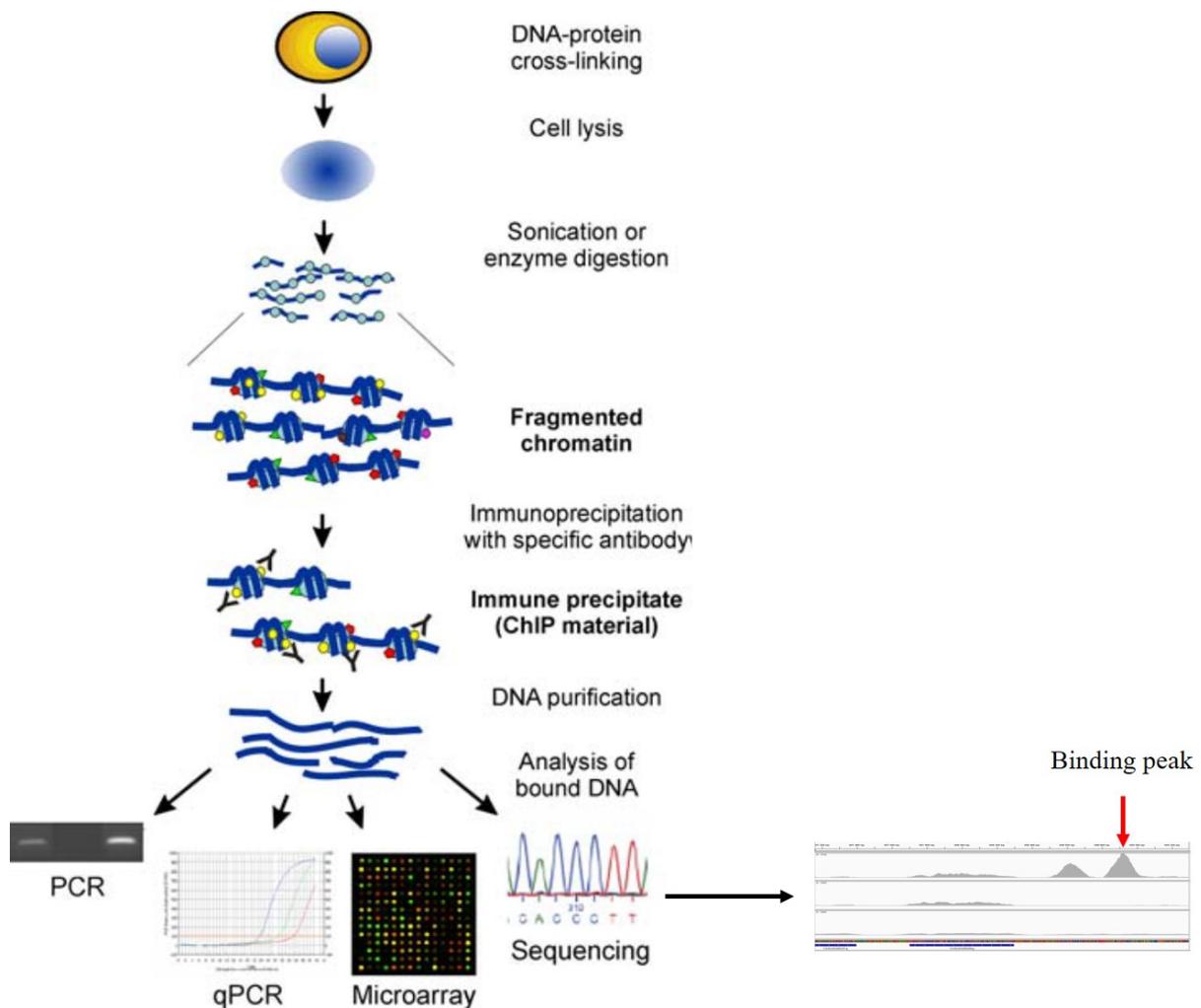


FIGURE 3.5. Chromatin Immuno-Precipitation protocol. This figure is adapted from Collas, 2010. It displays the steps to perform a ChIP assay, as well as the possible readouts of the experiment. Sequencing represents Next Generation Sequencing. The part on the bottom-right of the figure is a visualization of sequenced reads mapped on a reference genome and the detection of a peak, corresponding to a binding site.

cisplatin, dimethylarsinic acid, potassium chromate) or types of light (UV light and lasers) can be used (Das et al., 2004). However, most of them lose the biggest advantage of formaldehyde : its crosslinking is easily reversible. Formaldehyde also has another flaw : it can capture proteins that are close to DNA but not directly bound on it (for example, proteins interacting with a complex directly bound to DNA), producing false positive results. It is one of the reasons why O’Neill et al., 2003 developed Native-ChIP, which avoids using formaldehyde. Nevertheless, it also has disadvantages (Jordán-Pla et al., 2018), such as possible movements of proteins along DNA during lysis/sonication and inefficient capture of weak DNA-binders, such as TF. This explains why we used formaldehyde in our experiments.

- The IP relies a lot on the antibody that is used. A high affinity antibody will give impressive

results, while a bad counterpart won't properly precipitate DNA. Thus, the antibodies need to be validated. Wardle et al., 2015 reviewed this topic and proposed some techniques to appraise this reagent. But even with a proper validation, the antibodies may not efficiently immuno-precipitate all of the antigen from one experiment to the next. The use of specific antibodies also implies that everytime someone wants to study a new proteins using ChIP, he has to get a new proper antibody, or create one if it is not already existing. Unfortunately, this procedure is very long and expensive. A more affordable and easier idea is to add a peptide tag on the protein of interest. In this case, the antibody is always the same, but the tag can be placed on different proteins. The main condition for such a system is that the tag should not change the behavior of the protein (for example, an overexpression of the tagged protein may cause non-specific interactions with genomic sites or protein complexes).

- Even if they are perfectly adapted to an experiment, the antibodies can still provoke false positives by binding on non-specific proteins or directly on DNA. Besides the input control, another control, called "mock", can be used. It relies on performing ChIP on untagged cells. This way, it allows the investigator to find the DNA regions that are always bound (directly or not) by mistake and decrease the number of false-positives.

Despite, ChIP is still an incredible technique with several strong advantages : it can be performed *in vivo*, thus allowing the dynamic visualization of DNA-interacting proteins in their natural context, through the characterization of their association with specific genomic targets. This method can be applied to virtually any protein, as long as you have the right antibody, or the right epitope to tag the protein. ChIP, and especially ChIP-seq, gives extremely precise results on binding sites.

Yet, another important question remains : how is analyzed the immuno-precipitated DNA ? There are two main ways to analyze the ChIP products. First, the DNA can serve as a template for precise mapping by quantitative-PCR using primers for specific regions of interest. Second, the DNA can be used for high throughput analysis. It will be either by hybridization to a microarray covering a subset or the complete genome of the organism (ChIP on chip) or directly by sequencing (ChIP-seq). The high throughput approach is more expensive and takes more time to perform than the qPCR, but it is much more precise and provides a huge amount of data, that allow to precisely determine the binding sites of a TF as well as mutations in these sites. A more detailed comparison of ChIP-chip and ChIP-seq can be found in Hoffman et al., 2009.

We selected the ChIP-seq approach for the Candihub project. The sequencer we used is an Illumina NextSeq 500, which produces 75 bp single reads. We also chose to multiplex several samples (*i.e.* gather several samples in a single sequencing lane). To multiplex the samples, we used the Illumina Multiplexing Sample Preparation Oligonucleotide Kit. The general principle is to add barcodes to all the DNA fragments of a sample, using one specific barcode for each sample. The multiplexing allowed us to decrease the sequencing costs and avoided a uselessly big number of reads for a single sample : given the small size of *C. glabrata* genome, we didn't need 140 millions of 75 bp reads (approximately 2500 times the length of the genome) for a single sample. Hence, as all multiplexed data provided by a sequencer, the reads and the sequences needed to be cleaned before being analyzed for TF binding sites. We trimmed the barcodes and adapters off the reads using Cutadapt. Cutadapt was also used to trim sequence with a poor quality. Then, we mapped the reads on the reference genome of *C. glabrata* CBS138 using Bowtie. We chose to remove the reads mapping at multiple locations in the genome and the reads with strictly more than one mismatch. While this may generate false negatives, it assured us that no false positives were created by mapping the same read twice or more. Once all these steps were performed, the next step was to locate the binding sites.

The use of sequencing after ChIP experiments introduced new questions. In particular, one recurring question is : "How can we identify the binding sites with these data ?". When we look at the sequencing data using a visualization tool, for example Integrative Genome Viewer, it is possible to see an accumulation of reads at certain locations. These accumulations take the form of a peak (see Figure 3.5). Hence, a peak is an enrichment of specific DNA sequences which corresponds to a binding site of the studied protein. Peak detection is a crucial step in ChIP-seq data analysis. Choosing a suitable algorithm and optimized parameters to perform that task is critical as well. Several softwares were developed to automate the peak-calling procedure, *i.e.* the detection of peaks or binding sites. Thomas et al., 2017 determined the features defining a good ChIP-seq peak calling algorithm. To analyze our ChIP-seq data, we used bPeaks (Merhej et al., 2014). Its functioning is based on 4 parameters : the number of reads in the IP sample (P1), the number of reads in the control sample (P2), the value of log fold change between numbers of reads in IP and control samples (P3) and the sequencing coverage in both IP and control samples (P4). A peak is detected if it presents a high P1, P3, P4 and a low P2. The most important parameters are P3 and P4. In the plethora of available softwares, we chose bPeaks for three reasons :

- It was specially developed for small genomes. As a consequence, bPeaks exhibits a small computational time, as demonstrated by the benchmark of Merhej et al., 2014, who compared bPeaks

with three popular peak-calling programs : MACS (Y. Zhang et al., 2008), SPP (Kharchenko et al., 2008) and BayesPeak (Spyrou et al., 2009; Cairns et al., 2011).

- bPeaks is more precise in term of peak size : it usually gives peaks of 200 bases while the other softwares displayed peaks of several thousands of bases. The reason is that bPeaks analyses the ChIP-seq signals at the nucleotide level.
- Despite its simple mathematical model, it gives the same results than more complex algorithms such as MACS, SPP or Bayespeak.

To summarize, bPeaks is a good peak-caller that detects thinner peaks in a shortest period of time compared to other softwares, thanks to its simple mathematical model. However, one major default of this simple model is that bPeaks provides no statistical evaluation for each peak it detects. Hence, the addition of different types of information (such as transcriptomic data, phenotypic screening, presence of known binding sites in the peaks) is particularly useful to help evaluate the relevance of a peak.

3.3.3 Proof of concept of Candihub : the Yap network

Outside the Candihub project, I also performed transcriptomic analyses in the frame of the study of the regulatory interactions of the Yap family, which contains seven transcription factors. Using ChIP-seq and transcriptomic analyses, we reconstructed a part of the regulatory network associated to six of these factors and we were able to confidently define the binding sites of 5 TF, which allowed us to establish the strong conservation of DNA binding preferences between *C. glabrata* and *S. cerevisiae*. We also provided functional annotations for Yap1, Yap5 and Yap7 : Yap1 is mainly targeting genes in the oxidative stress response, oxido-reduction processes, chemical stress response and heme biosynthesis. Yap5 controls genes involved in iron-sulfur cluster biogenesis and iron homeostasis. The majority of Yap1 and Yap5 targets are conserved in *S. cerevisiae* and *textitC. albicans*. Despite its role as transcriptional repressor of nitric oxide oxidase (Merhej et al., 2015), we discovered that Yap7 binds genes in cytosolic and mitochondrial iron-sulfur assembly pathway, iron-sulfur cluster binding, oxidoreductases and heme metabolism. Overall, this data displayed the strong interconnection of Yap1, Yap5 and Yap7 which are situated in the middle of redox homeostasis, oxygen consumption and iron metabolism. We only had a few targets for Yap2, but they are involved in cadmium resistance, which let us think that this role is also conserved between *C. glabrata* and *S. cerevisiae*. Besides, we identified around 40 targets for Yap4/6, but we did not find any enriched functions. The enrichment of Sko1 binding motif among the target promoters might be a proof that Yap4/6 retained its role in osmotic stress, but this needs further

confirmation. All these results are presented in (Merhej et al., 2016), which can be found in Appendices 6.3.8. Given that the experimental techniques and the analysis softwares used in this paper are the same than in Candihub, this work also played the role of "proof of concept" on Candihub feasibility.

3.3.4 Goals of my PhD

My PhD took place in the frame of the Candihub project. I worked in Frédéric Devaux's team and thus focused on a single species, *Candida glabrata*. My role was to study 6 transcription factors essential in different stress responses by defining their sets of targets and their impact on gene expression in those stress conditions. When it was possible and in the focus of the lab, I investigated more thoroughly the TFs and their networks.

The TFs were chosen based on previous projects of the lab. The team analyzed and compared the transcriptomic responses of eight different yeast species (among *C. glabrata* and *C. albicans*) to the same pleiotropic stress : selenite. Selenite was chosen because it causes a pleiotropic stress, *i.e.* it triggers several different stress response pathways, including iron starvation, oxidative stress, unfolded protein response and general stress responses. Salin et al., 2008 showed that selenite is a suitable compound to study regulatory interactions between different stress response pathways. This project allowed the team to identify homologous TF which expression variations were identical (activated/repressed) when encountering stressful conditions. For this reason and because of the interests of the lab, we selected 6 TFs and decided to investigate Aft1, Aft2, Hap4, Hap5, Rox1 and Skn7. The role of these TFs in *S. cerevisiae* and sometimes in *C. glabrata* was presented previously. Rox1 and Skn7 were only studied by ChIP-seq and my results on these TFs will be mentioned in Results and Discussion Chapter 4. My study of Hap4 and Hap5 functions led to the publication presented in Results and Discussion Chapter 5. The roles of Aft1 and Aft2 were unraveled in the publication presented in Results and Discussion Chapter 6.

Results and discussions

Stress responses in *Candida glabrata* : a highly interconnected network

4.1 Introduction

As mentioned in the last part of the introduction, we performed several experiments of ChIP-seq on different transcription factors (Aft1, Aft2, Hap4, Hap5, Rox1, Skn7) in the frame of the Candihub project. This part will present an overview of all the ChIP-seq results obtained. Due to promising results, some of the transcription factors were studied more intensively and associated with transcriptomic data. These studies are presented in the following chapters (5 and 6).

4.2 Selection of the ChIP conditions

All our ChIP-seq experiments followed the same principle : the idea was to detect the binding targets of a TF when it is induced. To that end, we tagged the desired factors with a myc epitope to perform the ChIP (see Introduction Chapter 3.3.2) and we selected a range of conditions specifically designed to activate the TFs (and favour their binding as well) and the associated stress response. We used the pre-existing knowledge on the role of the factors to select growth conditions creating a stress and favouring the induction of the TF. When there was no publications on the role of a TF in *C. glabrata*, we looked for information in *S. cerevisiae*, assuming that given the closeness of the two species, the functions of the TF would be conserved. These conditions are summarised in Table 4.1.

The main reason for not applying a stress is that the TF is already naturally highly expressed, for example Rox1, which is highly expressed in normoxia. Aft factors are major regulators of the iron starvation response, hence we chose an iron chelator, BPS, to provoke iron-deprived conditions and

Transcription Factor	Condition	Stress
Aft1	BPS (500 μ M ; 30 min)	Iron starvation
Aft2	BPS (500 μ M ; 30 min)	Iron starvation
Hap4	YPD	None
Hap4	Glycerol (2% ; 4h)	Non-fermentative media
Hap5	YPD	None
Rox1	YPD	None
Skn7	NaCl (1 M ; 20 min)	Osmotic stress
Skn7	H ₂ O ₂ (0.4 mM ; 30 min)	Oxidative stress

TABLE 4.1. Summary of the conditions used to perform ChIP-seq. BPS stands for BathoPhenanthroline diSulfonic acid, an iron chelator. YPD is rich growth medium, which is equivalent to an absence of stress. Glycerol is the same media than YPD, except that glucose was replaced by glycerol.

induce them. Hap4 and Hap5 were tested in rich growth conditions (YPD) because *C. glabrata* still uses respiration in glucose, even it favours fermentation. This explains why we also tested Hap4 in glycerol : the absence of glucose forces the cells to use the respiratory pathway and induces Hap4, the major regulator of Hap5 and the CBC in this case. Finally, Skn7 has two main roles, in the osmotic and the oxidative stress responses, which is why we used hydrogen peroxide, one of the major cause of ROS for *C. glabrata*, and salt, which creates a strong imbalance in osmotic pressure between the outside media and inside the cells.

4.3 Sequencing of the immuno-precipitated DNA

Once the ChIP experiments were performed, we used next generation sequencing as a readout. A sequencing platform added barcodes to our samples, which allowed to multiplex them in a sequencing lane, thus decreasing the costs, and avoided us to recover too many sequences for a single sample. We used an Illumina NextSeq 500 sequencer. Table 4.2 recapitulates the number of reads we obtained for each sample.

We can see (Table 4.2) that the total number of reads vary a lot between the different samples. It may come from the differences of quantity in immuno-precipitated DNA. This differences come from discrepancies in ChIP efficiency. After sequencing, we had to process and filter the reads. We used Cutadapt to remove the adapters and the barcodes, as well as sequences with a poor quality. Hence, the length of the reads went from 75 bp to a length comprised between 50 and 63 bp. We used Bowtie to map the reads and index them. These steps usually cause losses of reads, and these experiments were no exception. Table 4.2 presents the reads lost at each step and the final number of reads that can be used for interpretation. Unmapped reads are quite low. They may be caused by poor DNA quality at the end of

Sample	Total number of reads	Unmapped reads	Reads with multiple mapping	Mapped reads
Aft1	23 504 142	4 019 726	3 581 407	15 897 225 (67,6%)
Aft2	15 128 355	3 449 879	3 111 168	8 556 653 (56,6%)
Mock Aft1 and Aft2	39 488 011	5 479 152	7 730 683	26 260 347 (66,5%)
Hap4-YPD	36 555 113	8 976 363	1 075 015	26 503 735 (72,5%)
Mock Hap4-YPD	21 220 235	4 935 651	1 687 771	14 593 254 (68,8%)
Hap4-Glycerol	40 684 484	11 802 113	1 059 429	27 822 942 (68,4%)
Mock Hap4-Glycerol	37 265 683	12 383 552	1 076 635	23 805 496 (63,9%)
Hap5	36 964 004	4 974 366	12 518 716	19 392 382 (52,5%)
Rox1	38 952 784	6 522 398	4 976 088	27 449 314 (70,5%)
Mock Hap5 and Rox1	21 220 235	4 935 651	1 687 771	14 593 254 (68,8%)
Skn7-H ₂ O ₂	22 644 073	3 742 245	2 249 131	16 644 954 (73,5%)
Mock Skn7-H ₂ O ₂	24 755 146	8 596 053	2 335 959	13 817 917 (55,8%)
Skn7-NaCl	27 368 366	3 539 712	4 610 518	19 196 006 (70,1%)
Mock Skn7-NaCl	28 674 640	7 126 743	5 392 910	16 142 595 (56,3%)
Input	45 032 807	1 644 101	1 683 912	41 702 562 (92,6%)

TABLE 4.2. Summary of the reads number obtained after sequencing and processing. First column is the number of reads obtained with the sequencing. Second column provides the number of non-aligned reads. Third column is the number of reads that aligned at multiple locations in the genome. They were removed from the analyses. Last column presents the number of mapped reads and the percentage of mapped reads compared to the total number of reads.

the ChIP or by sequencing errors. Reads aligned multiple times are usually sequencing artefacts. Despite the reads losses, the final number of reads are substantial and largely enough to study TFs binding on a small yeast genome (12.3 Mb).

4.4 Peak-calling and identification of the targets

After the reads filtering, the next step to perform is the peak-calling. As mentioned earlier, we used the bPeaks software. For each set of data, we first launched a "calibration" run. It means that we gave bPeaks several ranges of parameters in order to evaluate the number of peaks found with each set of parameter. Then, we chose a set of parameters (usually a set that gave a median number of peaks, compared to peaks found with the most stringent or the loosest sets or parameters) and evaluated the quality of the peaks found by checking them on IGV (Thorvaldsdóttir et al., 2013). This visual inspection allowed us to check for the presence of false-positives (such as peaks on highly expressed ORFs and tRNA). If necessary, *i.e.* if a set of parameters gave too many false peaks or a restricted number of peaks, we relaunched the peak-calling with other parameters. Once a set of parameters was fixed, we performed two peak-callings using this set of parameters : we compared the IP sample to the input control, and the IP sample to the mock control. This gave two lists of peaks for each sample. We kept only the peaks appearing in both peak-calling procedures. This method helped us to achieve a peak calling with few false-positives and a reasonable number of peaks. The next step was to assign promoters to the peak lists. Table 4.3 displays the number of peaks and potential targets for each transcription factor.

Transcription Factor	Number of peaks	Potential targets	GO enrichment
Aft1	109	148	Iron homeostasis
Aft2	63	88	Iron homeostasis Oxidative stress response
Hap4-Glycerol	314	437	Cellular respiration Oxido-reduction processes
Hap5	113	154	Cellular respiration
Rox1	370	473	Filamentous growth regulation
Skn7-H ₂ O ₂	193	267	Oxidative stress response
Skn7-NaCl	156	200	No enrichment)

TABLE 4.3. Peaks, targets and GO enrichment of each transcription factor. First column is the number of peaks found with bPeaks. Second column provides the number gene promoters associated to those peaks. Last column presents the GO processes enriched in the genes list. GO enrichment were evaluated thanks to the GO Term Finder tool of the CGD (<http://www.candidagenome.org/cgi-bin/GO/goTermFinder>).

The table 4.3 reveals that peaks number and potential targets are always different. It is caused by the fact that we can't always assign one promoter to a peak : if the peak is between two diverging promoters, it is impossible to choose one gene. To validate only one gene, supplementary informations are required, like transcriptomic data, binding motif presence... It is also possible, eventhough unlikely, that both genes are bound by the TF. There are huge variations between the peaks/potential targets of each TF. This may be caused by the importance and the different roles of a TF : a transcription factor with a restricted role will have less targets than a TF on top of the regulatory chain. Hence, it is not surprising to see a lot of potential targets for Hap4 and Rox1, while Aft2 has only a small set of targets. We also performed GO processes enrichment analyses that can be found in Table 4.3. Skn7-NaCl didn't present any enrichment because most of its targets were classified as "Unknown biological process" in the CGD. This analysis highlighted two interesting facts : 1. Aft2 displays an enrichment in oxidative stress response genes that was previously unknown in *Candida glabrata*. 2. Rox1 targets are enriched in genes of filamentous growth. Despite the fact *C. glabrata* rarely forms filaments, it is surprising because it resembles more *C. albicans* Rox1 than *S. cerevisiae*'s. CaRox1 was reported to play a role in regulation of filamentation while ScRox1 represses hypoxia genes during aerobiosis. Hence, it is likely this GO enrichment in filamentous growth among Rox1 targets comes from a wrong annotation transfer between *C. glabrata* and *C. albicans*. The other GO categories we found are in perfect accordance with the current knowledge on the TFs. Noteworthy, while it is not enriched for this category, Skn7-H₂O₂ has a lot of targets involved in cell wall maintenance and regulation, which reminds of one of the roles of Skn7 in *S. cerevisiae*.

4.5 Representing the Candihub network

When the targets of the transcription factors are defined, it is possible to use these lists to represent the interactions between TF and genes. We chose Cytoscape (Shannon et al., 2003) to perform that task. The network can be found in Figure 4.1.

To build this network, we used two sets of data :

- The ChIP-seq data of the Candihub project (Aft1, Aft2, Hap4, Hap5, Rox1, Skn7). When data for several conditions were available for a same transcription factor, we fused the lists of targets and removed the duplicates.
- The ChIP-seq data previously produced by the team during the study of the Yap family (Yap1, Yap2, Yap3b, Yap4-6, Yap5, Yap7) (Merhej et al., 2015; Merhej et al., 2016).

Hence, this network comprises 1057 nodes, including the 12 transcription factors and 1955 edges. The high number of nodes is not surprising given the wide diversity of the stress studied. The high number of edges (around twice the number of nodes) shows the strong interconnection between the different stress networks. Also, this network perfectly respects the scale-free model that is usually attributed to this type network (see Table 4.4) : there are a lot of weakly connected nodes ($\text{degree} \leq 2$, violet and red nodes) and a minority of strongly connected nodes ($\text{degree} \geq 6$, dark green and mid-green nodes), or hubs. Because we assembled several pathways in this network, we probably found 12 genes that are at the crossroads of several stress responses. These central genes (nodes with degrees 6 and 7) are *HEM3*, *SIT1*, *CCC1*, *APT2*, *ATG41*, *EFM1*, *TAL1*, *STR3*, *OYE2*, *CAGL0M11682g*, *CAGL0H07337g* and *CAGL0L09911g*. Interestingly, iron homeostasis is well represented with 3 genes (*HEM3*, *SIT1*, *CCC1*), which tends to further highlight the importance of that metal in yeast. The other genes are diverse and are involved in redox processes, autophagy, cell wall, biosynthetic processes. These genes are likely to have crucial role in the cell survival. Additionally, the low number of targets of Yap3b probably means that we didn't use the proper stress condition to activate the TF. Of note, Yap2 and Yap5 have no exclusive targets : all their targets are shared with one or several other transcription factors. This could mean that Yap2 and Yap5 are often acting as co-regulators (in the testes conditions, at least).

Some aspects of this wide network will be further discussed in the next chapters.

Node degree	1	2	3	4	5	6	7
Number of nodes	516	311	134	56	23	8	4

TABLE 4.4. Distribution of the node degrees. These data were obtained via target counting on Cytoscape.

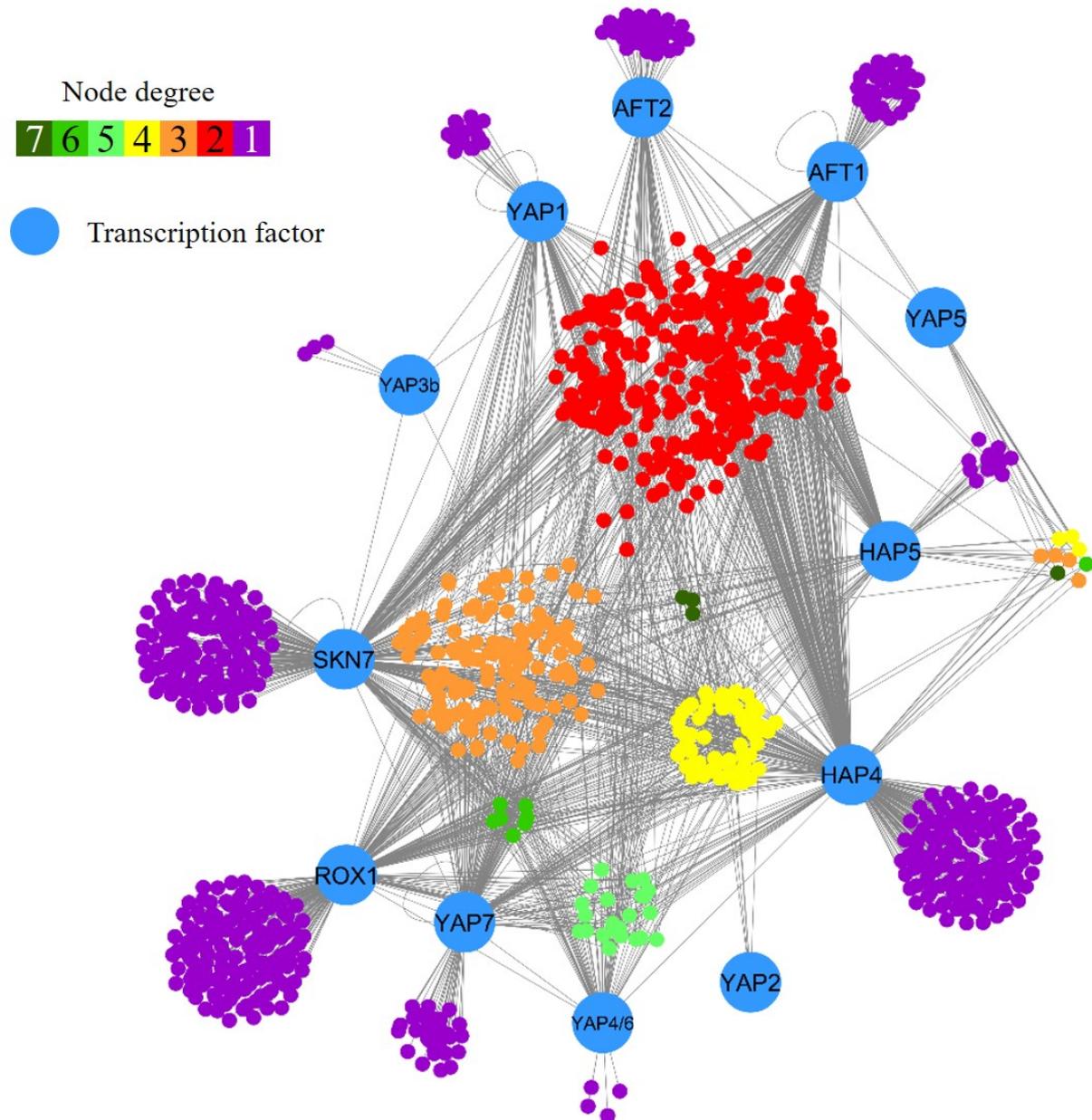


FIGURE 4.1. Network of interactions of *C. glabrata* stress responses. Transcription factors are the large light blue circles, target genes are the smaller colored circles. The color represents the node degrees. This network displays the data obtained during the Candihub project and the data obtained by the team during the study of Yap networks (Merhej et al., 2015; Merhej et al., 2016). This network was built thanks to Cytoscape (Shannon et al., 2003).

The CBC Impacts Respiratory Genes and Iron Homeostasis in *Candida Glabrata*

5.1 Introduction

As mentioned in Introduction Chapter 3, my PhD evolved out of the Candihub ANR project, which aimed to describe the transcriptional regulation networks involved in several stress responses in both *Candida albicans* and *Candida glabrata*. The project was especially focusing on the role of transcription factors in these responses, among which the Yeast activator protein (Yap) family of b-ZIP transcription factors. A previous study of the lab tackled the role of this family in *Candida glabrata* (Merhej et al., 2016). This family contains 7 members which are known to play an important role in a variety of stress responses such as oxidative stress, osmotic stress, heavy metals stress, nitrosative stress and iron stress. It was also posited that Yap5 might interact with the CCAAT binding complex (CBC) through a vestigial Hap4-like domain, the same way Yap5 orthologues (HapX proteins) are interacting with the CBC to act on iron homeostasis in *Saccharomycetales* such as *C. albicans*.

Following this hypothesis, we tried to unravel the role of the CBC in *C. glabrata*. To that extent, we performed DNA microarrays and Chromatin-ImmunoPrecipitation followed by sequencing to determine the targets of the CBC, and especially the targets of the Hap5 subunit which is known to be responsible for the DNA-binding ability of the CBC. We showed that the CBC plays a dual role in *C. glabrata*. It impacts the activation of respiratory genes through its interaction with Hap4 when yeast cells can't use the fermentation pathway. It is also crucial for the iron excess response through its interaction with Yap5 to activate iron consuming genes. Interestingly, Yap5 cannot bind to its target promoters without Hap5. Co-immunoprecipitation experiments implied an interaction between Yap5 and Hap5. Further experiments showed that the Hap4-like domain of Yap5 was likely to play a role in this interaction, because

mutations in this domain drastically weakened Yap5 binding to its targets as well as its interaction with Hap5.

Thus, Yap5 can be labeled as another regulatory subunit of the CCAAT-Binding Complex, which displays a new essential role in iron homeostasis. The role of the CBC seems to be conserved in species distant of *C. glabrata* such as *Schizosaccharomyces pombe*, even if the mechanisms of the CBC in iron homeostasis vary a lot between *S. pombe* (where Php4 binds the CBC that binds DNA) and *C. glabrata* (where the CBC and Yap5 both bind DNA and also interact together).

For this work, I performed the microarrays and the associated transcriptomic analyses. I performed the ChIP-seq experiments and the subsequent bioinformatic analyses (peak calling, cis-regulatory motifs enrichment analyzes). I also performed the q-PCR experiments and made their analyses.

5.2 Publication

SCIENTIFIC REPORTS

OPEN

The CCAAT-Binding Complex Controls Respiratory Gene Expression and Iron Homeostasis in *Candida Glabrata*

Antonin Thiébaut¹, Thierry Delaveau¹, Méline Benchouaia¹, Julia Boeri¹, Mathilde Garcia¹, Gaëlle Lelandais^{2,3} & Frédéric Devaux¹

The CCAAT-binding complex (CBC) is a heterotrimeric transcription factor which is widely conserved in eukaryotes. In the model yeast *S. cerevisiae*, CBC positively controls the expression of respiratory pathway genes. This role involves interactions with the regulatory subunit Hap4. In many pathogenic fungi, CBC interacts with the HapX regulatory subunit to control iron homeostasis. HapX is a bZIP protein which only shares with Hap4 the Hap4Like domain (Hap4L) required for its interaction with CBC. Here, we show that CBC has a dual role in the pathogenic yeast *C. glabrata*. It is required, along with Hap4, for the constitutive expression of respiratory genes and it is also essential for the iron stress response, which is mediated by the Yap5 bZIP transcription factor. Interestingly, Yap5 contains a vestigial Hap4L domain. The mutagenesis of this domain severely reduced Yap5 binding to its targets and compromised its interaction with Hap5. Hence, Yap5, like HapX in other species, acts as a CBC regulatory subunit in the regulation of iron stress response. This work reveals new aspects of iron homeostasis in *C. glabrata* and of the evolution of the role of CBC and Hap4L-bZIP proteins in this process.

The CCAAT-binding complex (CBC) is a heterotrimeric transcription factor which is conserved from fungi to vertebrates and plants and which specifically recognizes the CCAAT DNA motif. In yeasts, the three subunits of the CBC are called Hap2/3/5. In fungi, the Hap2/3/5 complex is sufficient to bind DNA but it requires a fourth subunit to regulate transcription of some of its target genes^{1–3}. Many roles have been attributed to fungal CBC but the most extensively studied are its requirement for the regulation of respiration in *Saccharomyces cerevisiae* on one hand, and its involvement in the regulation of iron homeostasis in many other fungal species (including several human pathogens such as *Candida albicans*, *Aspergillus fumigatus* or *Cryptococcus neoformans*) on the other hand (Supplementary File S1). In the model yeast *S. cerevisiae*, the regulatory subunit Hap4 allows the CBC to activate the expression of the respiratory pathway genes in the absence of glucose^{4–7} (Supplementary File S1). In this model, HAP4 is transcriptionally induced by non-fermentable carbon sources, while the expression and DNA binding of Hap2/3/5 are constitutive⁸. Hap4 indirectly binds DNA through its interaction with CBC. A conserved motif of 16 amino acids in the Hap4 sequence has been shown to be required for this interaction^{3,9,10}. In the pathogenic yeast *Candida albicans* and in many other fungal species (e.g. *Aspergillus fumigatus*, *Aspergillus nidulans*, *Cryptococcus neoformans*, *Fusarium oxysporum*, ...), the CBC plays an important role in iron homeostasis^{11–19}. This role is mediated by the HapX (named Hap43 in *C. albicans*) regulatory subunit. HapX acts by repressing the expression of iron consuming genes in iron starvation conditions and by activating these genes in iron excess^{11,13,17,18,20}. HapX proteins have few similarity with Hap4 except for the 16 amino acids domain (called Hap4Like or Hap4L) which is required for their interaction with Hap5^{12,18,21,22}. In addition, they also have a bZIP DNA binding domain similar to the one found in the oxidative stress response factor Yap1^{12,18,21–23}. In Yap1, this domain is responsible for the specific recognition of the YRE (Yap Response Element) DNA motif (TTACTAA). The role of

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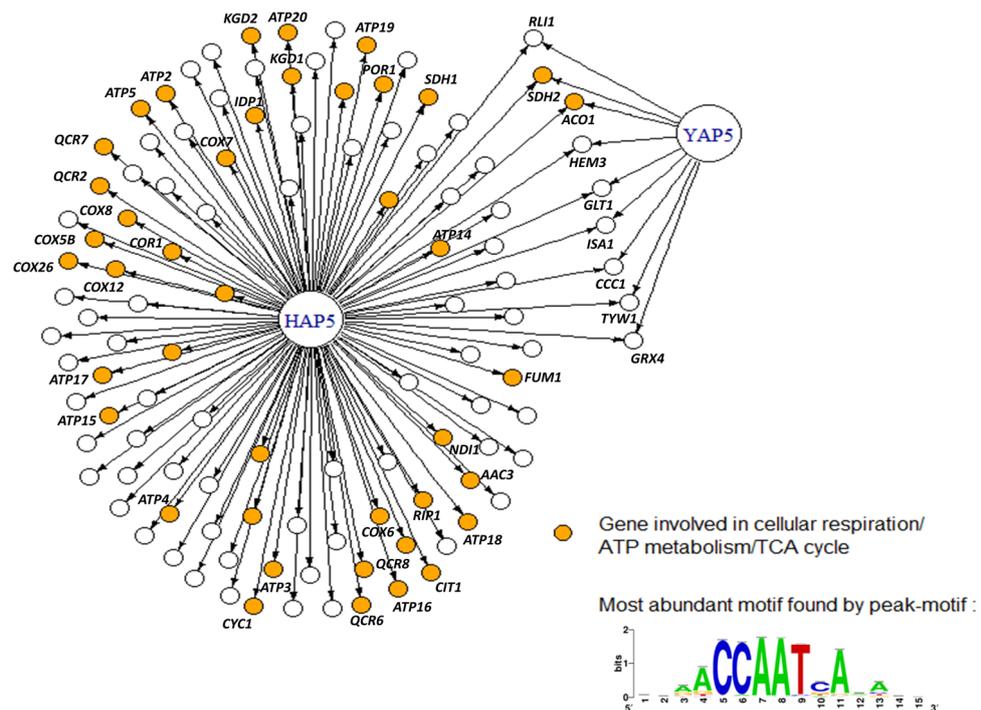


Figure 1. The Hap5 network in *Candida glabrata*. An arrow indicates a potential regulatory interaction based on ChIP-seq. The color of the targets indicates their belonging to respiratory pathways (yellow) or not (white). The most enriched DNA motif in ChIP peaks is represented at the bottom right. The Yap5 data are from ref. 29. The gene names indicated are those of the *S. cerevisiae* orthologs (according to the CGD database, www.candidagenome.org). For the sake of clarity, only the names of the genes which are discussed in the main text are indicated.

this domain in HapX proteins remains to be elucidated since HapX and Hap43 clearly bind CCAAT motifs indirectly through their interaction with CBC, rather than YREs^{11, 20, 24}. However, HapX also contributes to the DNA binding specificity of the CBC-HapX complex in *A. nidulans* and the bZIP domain is absolutely required for the regulation of transcription by Hap43 in *C. albicans*^{18, 25}.

In *S. cerevisiae*, in contrast to *C. albicans* and *Aspergillus sp.*, CBC does not seem to contribute to the global iron starvation response²⁶, but its role in iron homeostasis has actually been poorly investigated in this species²⁷ (discussed in refs 28, 29). A potential ortholog of Hap43/HapX, named Yap7, has been identified in *S. cerevisiae*^{18, 23}, but it is not directly involved in iron starvation or iron excess responses²¹.

In the present study, we analyzed the role of the CBC in the yeast *Candida glabrata*, a human pathogen which is evolutionary much closer to *S. cerevisiae* than to *C. albicans*³⁰. We identified the targets of Hap5 using chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq) and transcriptome analyses. We found that Hap5 binds many genes involved in respiration and positively controls their expression. As in *S. cerevisiae*, this function requires the Hap4 regulatory subunit. More surprisingly, we observed that Hap5 directly controls the iron stress response mediated by the Yap5 bZip transcription factor. We showed that Yap5 cannot bind the promoter sequence of *GRX4* in the absence of Hap5 and co-immunoprecipitation experiments suggested that Hap5 interacts with Yap5. Interestingly, Yap5 contains a sequence which resembles the N-terminal part of the Hap4L domains of Hap4 and HapX. Full deletion or substitutions of the conserved amino acids in this sequence severely impaired Yap5 binding to its targets and its interaction with Hap5. Our results revealed a key role of CBC in the iron stress response of *C. glabrata* and identified Yap5 as a new CBC regulatory subunit.

Results

Hap5 targets and controls both the respiratory genes expression and the Yap5 mediated iron stress response. To identify the targets of CBC in *C. glabrata*, we analyzed the DNA binding pattern of Hap5 by ChIP-seq. We chose this subunit because it was shown in other species to be essential for the assembly, the DNA binding and the activity of CBC^{31, 32}. Moreover, Hap5 is the CBC subunit which recruits Hap4 through a Hap4 recruitment domain which is present only in fungi³². We performed ChIP-seq on exponentially growing cells in rich glucose media. The peak calling procedure identified 113 bound promoters and 154 potential target genes (Supplementary File S2). Bioinformatic analyses using the peak motif software unambiguously identified the CCAAT box as the most enriched motif in the ChIP peaks, being present in 85% of them (Fig. 1, Supplementary File S2). Gene Ontology analyses of the list of potential target genes showed a strong enrichment in genes encoding proteins involved in cellular respiration ($p = 1.6 \times 10^{-23}$), ATP metabolism ($p = 6.1 \times 10^{-29}$), oxido-reduction processes ($p = 1.7 \times 10^{-20}$) and TCA cycle ($p = 6.2 \times 10^{-10}$). Half of the targeted promoters were associated to a gene encoding a mitochondrial protein ($p = 3.4 \times 10^{-18}$). These included genes encoding subunits

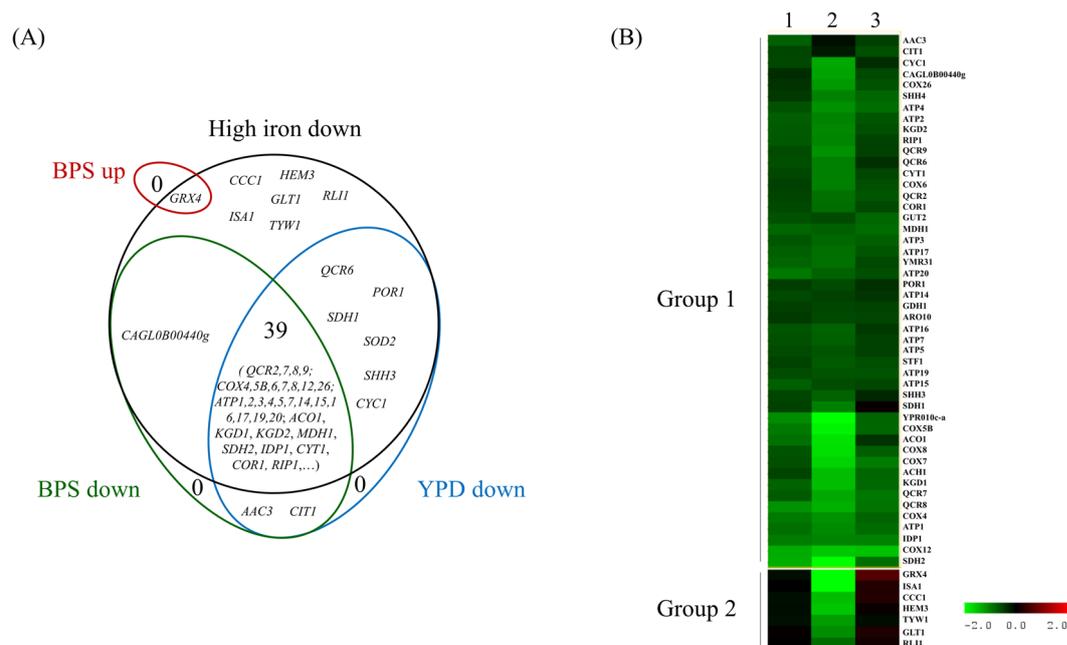


Figure 2. Transcriptome analyses of Hap5 impact on gene expression. The wild type and *hap5Δ* strains were grown in three different conditions (rich media, iron excess or iron starvation) and their transcriptomes were compared using microarrays. **(A)** Venn diagram representing the overlaps between the lists of Hap5 ChIP targets being significantly down regulated compared with wild type in YPD (blue line), BPS (green line) or iron excess (black line). The red line includes the only Hap5 ChIP target (*GRX4*) which was significantly up-regulated upon BPS treatment. The gene names are from the *S. cerevisiae* orthologs, when available. **(B)** Eisengram of the expression profiles of the genes from the Venn diagram. The values used are \log_2 of *hap5Δ*/wild type expression ratios. The color scale is indicated. The conditions used are 1: YPD; 2: iron excess (2 mM FeSO_4 for 30 minutes); 3: iron starvation (0.5 mM BPS for 30 minutes).

of the respiratory complexes (*COX*, *ATP* and *QCR* genes, *COR1*, ...), mitochondrial transporters (*AAC3*, *POR1*, ...) and enzymes of the TCA cycle (*ACO1*, *SDH2*, *KGD1*, *KGD2*, ...) (Fig. 1).

Another remarkable feature of the ChIP results is that all the targets previously identified for Yap5²⁹ were also bound by Hap5 (Fig. 1). These correspond to 9 genes induced by Yap5 in iron excess conditions and encoding the glutaredoxin *Grx4*, the iron-sulfur cluster containing enzymes *Aco1*, *Tyw1*, *Sdh2*, *Rli1* and *Glt1*, the iron-sulfur cluster maturation enzyme *Isa1*, the heme biosynthetic enzyme *Hem3* and the iron vacuolar transporter *Ccc1*.

To measure the impact of Hap5 on the expression of its targets, we performed transcriptome analyses comparing gene expression between a *hap5Δ* mutant and wild type cells. Because our ChIP-seq results showed an interaction between Hap5 and the targets of Yap5, we performed these transcriptome experiments on cells grown in three different conditions: standard rich media, iron excess and iron starvation. Among the 113 target promoters identified by ChIP-seq, 55 showed altered expression of one of the corresponding gene in at least one of the three tested growth conditions (Fig. 2A). Two main types of targets were distinguished based on their expression profiles (Fig. 2B). The first one included 48 genes which expression was diminished in the mutant in at least two of the three tested conditions (Fig. 2). Among them, 39 showed lower expression in all three conditions (Fig. 2A). This group of « constitutively » affected targets included mainly genes encoding proteins related to respiration and mitochondrial activity. The second group included 7 genes which expression was specifically impaired in iron excess conditions (Fig. 2). These genes were all previously shown to be regulated by Yap5²⁹. In this group, *GRX4* has a special status since its expression is down regulated in the Hap5 mutant in iron excess conditions but up-regulated in the same mutant upon iron starvation (Fig. 2A). Actually, this *GRX4* expression pattern is exactly the same as the one previously described for *yap5Δ* mutants²⁹. ChIP-Q-PCR analyses showed that the binding of Hap5 to *GRX4* was constitutive and independent of iron availability (Supplementary File S3), as previously described for Yap5²⁹.

Hap5 cooperates with Hap4 in the regulation of respiratory genes expression and with Yap5 in the iron excess response. Our ChIP-seq and transcriptome experiments demonstrated a dual role for Hap5 in the constitutive expression of respiratory genes on one hand, and in the iron excess activation of Yap5 targets on the other hand. The first role is consistent with what was previously shown for CBC in *S. cerevisiae*. In this species, Hap5 requires the regulatory subunit Hap4 to control the expression of respiratory genes in the absence of glucose^{2, 5-7}. To clarify the potential interplays between Hap5, Hap4 and Yap5 in *C. glabrata*, we performed Q-PCR analyses of *ATP2* (group 1 in Fig. 2B) and *GRX4* (group 2 in Fig. 2B) expression in wild type, *hap5Δ*, *hap4Δ* and *yap5Δ* cells grown either in glucose, glycerol (a non-fermentable carbon source) or iron excess. As observed in our transcriptome analyses, *ATP2* levels are decreased in the *hap5Δ* mutant in all three growth conditions examined. Remarkably, the same pattern was observed for the *hap4Δ* mutant, while *ATP2* levels were independent on the presence of *YAP5*

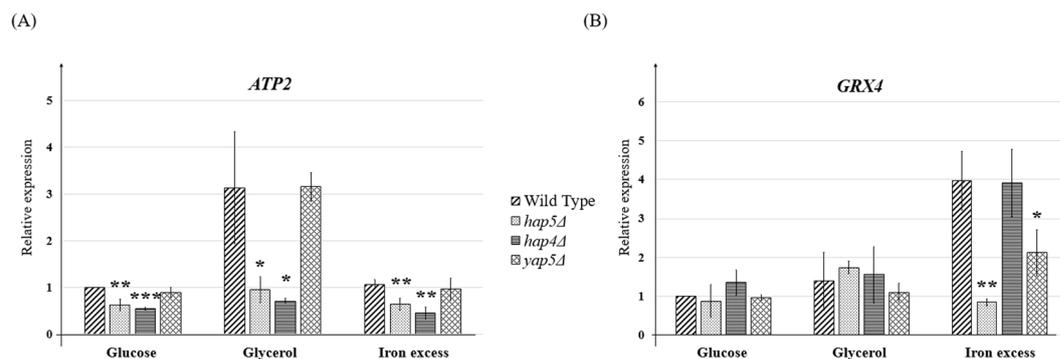


Figure 3. Analyses of the impact of Hap5, Hap4 and Yap5 on *ATP2* and *GRX4*. The relative expression of *ATP2* (A) and *GRX4* (B) was measured by Q-RT-PCR in wild type, *hap5Δ*, *hap4Δ* and *yap5Δ* strains grown in glucose, glycerol or iron excess. The values represent the expression levels of the *ATP2* or *GRX4* genes relative to *ACT1* (used as an internal control) and to the wild type grown in glucose. The experiments were performed three times on biologically independent samples. Error bars represent the pearson standard deviation. A t-test was performed to compare, for each growth condition, the mutants to the corresponding wild type. The results of the test are indicated by the stars as follows *p < 0.05, **p < 0.01, ***p < 0.001.

(Fig. 3A). Similar results were obtained for another respiratory gene (namely *COX12*, Supplementary File S4). In contrast, the basal levels of *GRX4* remained unaffected in all three mutants but its induction by iron excess was totally abolished in the *hap5Δ* and *yap5Δ* mutants, while being unaffected by the deletion of *HAP4* (Fig. 3B).

To further investigate the relationships between Hap5 and Yap5, we compared the Hap5 and Yap5 ChIP-peak positions and sequences. We observed that the Yap5 and Hap5 ChIP peaks were at the same locus. Moreover, seven out of nine Yap5-Hap5 ChIP targets had both a YRE motif and a CCAAT box in their Yap5 and Hap5 peaks (Supplementary File S5). Of note, *ACO1* and *SDH2*, which contain only a CCAAT-box in their promoters, are also the only two Hap5-Yap5 targets which behaved as respiratory pathway genes and not as iron excess response genes in the transcriptome experiments presented in Fig. 2. Strikingly, the YRE and CCAAT box found in the 7 other targets were always located very close to each other, with a spacing varying from 10 to 14 base pairs between the two motifs (Supplementary File S5). A genome-wide search for co-occurrences of CCAAT-box and YRE with a spacing from 10 to 14 base pairs showed that this situation is very unusual, being present in only 28 gene promoters in *C. glabrata*. This conserved and unusual spacing between the two motifs in 7 out of 9 of the promoters which were co-regulated by Hap5 and Yap5, strongly suggested that Yap5 and Hap5 had to interact to control the expression of those genes.

We next performed ChIP analyses of Yap5 binding to *GRX4* in wild type and *hap5Δ* cells (Fig. 4). We observed that the absence of Hap5 totally abolished Yap5 binding to its target. In a previous work, we noticed that Yap5 contains a degenerated but still recognizable Hap4L motif just upstream of its bZIP domain²¹. This motif corresponds to the N-terminal half of the canonical Hap4L domain (Fig. 4A). To assess the potential role of this sequence in the interaction between Yap5 and Hap5, we mutagenized the conserved part of the Yap5-Hap4L domain and tested by ChIP the capacity of the mutated Yap5 versions to bind *GRX4* in the presence of a wild type Hap5 (Fig. 4). First, we deleted the 11 amino acids of the conserved part of Yap5-Hap4L domain. In *C. albicans*, a similar deletion in the Hap4L-bZIP protein Hap43 was shown to abolish its capacity to regulate iron homeostasis¹⁸. The corresponding Yap5 mutant protein (*yap5-Hap4LΔ*) was normally expressed (Supplementary File S6) but unable to bind *GRX4* promoter (Fig. 4B). Next, we performed amino-acid substitutions at positions of the Yap5-Hap4L domain that are conserved in the Hap4 protein (S33P and K34E) (Fig. 4A). These substitutions were chosen because they were shown to abolish the activity of Hap4 in *S. cerevisiae* and therefore are likely to affect residues essential for the Hap4L-Hap5 interaction⁹. These mutations severely diminished the binding of Yap5 to *GRX4* compared to the wild type version (Fig. 4B, Supplementary File S6).

These results strongly suggested that Yap5 and Hap5 had to interact to control the expression of their common targets. To support this hypothesis, we performed co-immunoprecipitation (co-IP) experiments by tagging the chromosomal version of Hap5 with protein A and transforming the resulting strain with a plasmid bearing the wild type or the mutated versions of Yap5-myc under the control of the native *YAP5* promoter. IP samples obtained by immunoprecipitating Hap5-ProtA were then analyzed by western blot using an anti-myc antibody (Fig. 4C) or an antibody with a high affinity for Protein A (Supplementary File S7). A clear band corresponding to Yap5-myc was observed in the Hap5-ProtA IP with the wild type version of Yap5, but not with the two Yap5 versions mutated for the Hap4L domain (Fig. 4C). This difference was not due to differences in the input samples or in the IP efficiency since the input Yap5-myc signals were not lower for the mutants compared with the wild type and since the input and IP Hap5-ProtA signals were equivalent in all lanes (Fig. 4C and Supplementary File S7). This result supports the model in which Yap5 interacts with Hap5 through its Hap4L domain.

Discussion

In this work, we identified a part of the transcriptional network associated with CBC, more particularly the Hap5 subunit, in the pathogenic yeast *C. glabrata*. As mentioned in the introduction, the main role described for CBC in the model yeast *S. cerevisiae* is the activation of respiratory pathway genes in the absence of glucose. This also involves the regulatory subunit Hap4. Very recently, it was reported that *hap5Δ* and *hap4Δ* strains have a severe

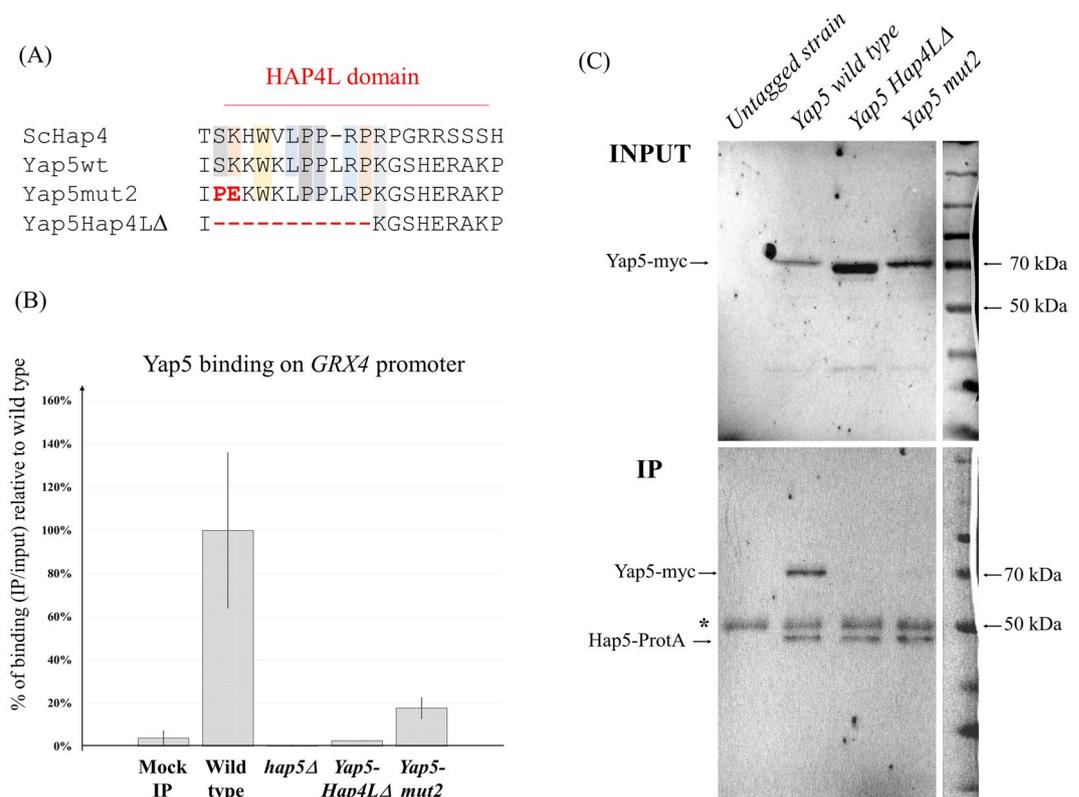


Figure 4. Molecular basis of the Yap5-Hap5 interaction. (A) Multiple alignments of the Hap4L domains of Hap4 (from *S. cerevisiae*), wild type *C. glabrata* Yap5, Yap5-Hap4LΔ and Yap5-mut2. For the latter, the substitutions are highlighted in red. (B) ChIP-QPCR was performed on strains expressing a myc-tagged Yap5 in presence (wild type) or absence (*hap5*Δ) of *HAP5* and on strains expressing the two different Yap5 mutant versions. All strains were grown in YPD. The values represent the IP/Input ratios of the *GRX4* promoter relative to the enrichment of the *YHB1* promoter (used as an internal control), expressed as a percentage of the enrichment obtained for the wild type Yap5. The experiments were performed twice on biologically independent samples. Error bars hence represent the standard error of the mean. (C) Western blot analyses of the co-immunoprecipitation experiments using Hap5-Protein A as bait and wild type or mutated versions of Yap5-myc as prey. Upper panel: input samples (INPUT), lower panel: immunoprecipitated samples (IP). Immunoblotting was performed with a mouse anti-myc antibody (Roche). The Yap5 protein is fused to 13 c-Myc epitopes and the corresponding band is expected at 65 kDa. The Hap5-Protein A fusion is expected at 45 kDa and is also detected by the anti-myc antibody (although with a low affinity), because Protein A non-specifically interacts with IgG. Note the similar intensity of the Hap5-ProtA bands in the IP, which indicates that the IP efficiency was equivalent from one lane to another. The star indicates the 50 kDa band corresponding to the large chain of the anti-Mouse antibodies used for the IP. The co-immunoprecipitation experiment was performed twice on biologically independent samples and gave consistent results. The ladder on the right was copied and pasted from the white light image of the membrane. Immunoblotting of the same membranes with rabbit IgG-HRP polyclonal antibody (PAP; code Z0113; Dako), which has a high affinity for Protein A, can be found in Supplementary File S7.

growth defect in non-fermentable carbon sources in *C. glabrata*, indicating that this role may be conserved in this species³³. Consistently with this observation, our genome-wide analyses showed that Hap5 positively controls the expression of many mitochondrial protein encoding genes, including subunits of the respiratory complexes and enzymes from the TCA cycle. We also showed that Hap4 is required for this regulation. Hence, this role in cellular respiration seems to be perfectly conserved between *S. cerevisiae* and *C. glabrata*.

More unexpectedly, we demonstrated a role of the *C. glabrata* CBC in iron homeostasis. Hap5 was found to bind the promoters of the genes of the Yap5-dependent iron stress regulon (i. e. *GRX4*, *ISA1*, *RLI1*, *HEM3*, *TYW1*, *GLT1*, *CCCL1*, *ACO1* and *SDH2*) and is necessary for their induction in iron excess conditions (Figs 1, 2 and 3). Hap5 also plays a role in the negative regulation of *GRX4* in iron starved conditions (Fig. 2). This is reminiscent of the situation extensively described in *C. albicans* and many other fungal pathogens, in which the CBC plays an important role in iron starvation and iron stress responses. Hence, the CBC of *C. glabrata* seems to combine features of *S. cerevisiae* CBC (positive regulation of respiration) and *C. albicans* CBC (positive regulation of iron stress response) (Fig. 5). Interestingly enough, this is not the only example of this kind. It was recently shown that the iron starvation regulation in *C. glabrata* relies on a hybrid network which combines the “*S. cerevisiae*-like” Aft1 activity with the “*C. albicans*-like” Sef1 transcription factor³³.

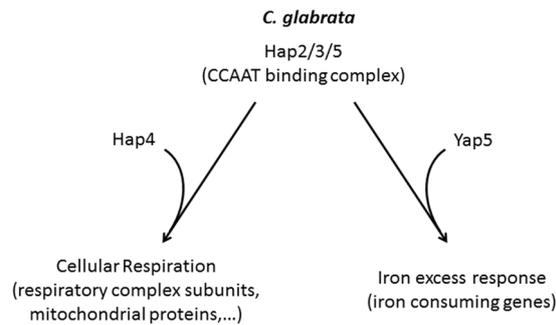


Figure 5. A dual role for CBC in *Candida glabrata*. CBC plays a dual role in the control of cellular respiration (together with the regulatory subunit Hap4) and of the iron stress response mediated by the Yap5 transcription factor.

In *S. cerevisiae* and *C. glabrata*, iron stress response has been shown to be controlled by the Yap5 bZIP transcription factor^{21, 34–36}. This regulation absolutely requires the direct binding of Yap5 to YRE motifs (TTACTAA) in the promoters of these genes^{29, 34, 35}. We showed here that Hap5 is also necessary for this regulation to happen. More precisely, Yap5 is unable to bind and activate its targets in the absence of Hap5 (Fig. 4). Remarkably, the YRE and CCAAT motifs in the promoters of the Hap5-Yap5 iron stress regulon exhibited a conserved spacing of 10 to 14 base pairs, suggesting that the proximity of the two transcription factors on DNA is important for their activity. We previously noticed that Yap5 contains a Hap4L like domain which actually corresponds to half of the canonical Hap4L domain found in published CBC regulatory subunits such as Hap4 in *S. cerevisiae* and Hap43/HapX in pathogenic fungi²¹. Remarkably, this motif was not found in a global search for Hap4L-bZip bipartite domains¹⁸ and did not pass the statistical cut-off in a Hap4L search using the Pfam database²¹. Yet, partial deletion or point mutations of this domain severely reduced Yap5 binding to *GRX4* and this domain was necessary for the Yap5-Hap5 interaction detected in co-IP experiments (Fig. 4). These results suggest that, like HapX and Hap43, Yap5 needs to interact with the CBC to control iron homeostasis and that the conserved amino-acids of its truncated Hap4L domain play a key role in this interaction. In this model, Yap5 would function as a CBC regulatory subunit of a special kind, since it has its own DNA interaction which is as important for its action than its interaction with CBC and since its Hap4L domain significantly diverged from the canonical version.

This observation shed new interesting light on the conservation of the role of CBC in fungi and on the divergence of the associated regulatory mechanisms (Fig. 6). In the fission yeast *Schizosaccharomyces pombe*, iron homeostasis is regulated by Php4 which negatively controls the expression of iron consuming genes in iron starved cells by binding to the CBC through its HAP4L domain and by sensing iron by the intermediate of Grx4 (reviewed in ref. 15). As far as we know, Php4 does not directly bind DNA and does not contain a bZIP domain. In many pathogenic and non-pathogenic fungi (*Aspergillus* sp., *Cryptococcus neoformans*, *Fusarium* sp., *Candida albicans*), HapX/Hap43 proteins negatively regulate iron consuming genes in iron starvation and positively regulate them in iron excess^{12, 13, 16–18, 20}. They directly sense iron concentration through conserved cysteine rich motifs²⁰. Gene expression regulation by HapX involves both binding to CBC and direct interaction with a DNA consensus (TGAC) which is close to a half-YRE site and which may rely on the bZIP domain of HapX^{11, 20, 25}. The Yap5 functioning described here is very close to HapX, except that Yap5 binds a full YRE site^{29, 34, 35}. Interestingly, Yap5 senses iron excess through a cysteine rich domain (CRD) which is very similar to the CRD of HapX³⁷. Moreover, like HapX, *C. glabrata* Yap5 can act both as a repressor or an activator, depending on iron availability²⁹. Then, CBC's role in iron homeostasis is globally conserved (except that its role in iron starvation in *C. glabrata* is marginal and restricted to the repression of *GRX4*) but its mechanisms of action have diverged from a Hap4-like functioning in *S. pombe* to a fully cooperative DNA binding model in *C. glabrata*. In this model, the CBC-HapX case would be an intermediate situation, in which HapX also contribute to DNA binding specificity and affinity but in which the interaction with CBC is predominant (Fig. 6).

This model also provides keys to the answer to a long standing question, which is the basis of the Yap DNA binding specificity. Yap1, Yap2, Yap5 and Yap7 all recognize TTACTAA as their favorite consensus binding site. Yet, ChIP-seq and ChIP-chip analyses revealed that they have quite different sets of targets^{29, 38}. The strong dependence of Yap5 DNA binding on the presence of both an YRE motif and of a CCAAT motif bound by Hap5 at a distance of 10–14 base pairs may partly explain its target specificity compared to the other Yap proteins, since this particular co-occurrence is very unusual in the genome of *C. glabrata*.

Additional roles of CBC have been described in fungi. For instance, in *C. albicans* and in *S. cerevisiae*, CBC has a role in the transcription activation of genes required for growth on poor nitrogen sources (e.g. *GDH1*, *GDH3* and *ASN1* in *S. cerevisiae*; *MEP2* and *SAP2* in *C. albicans*)^{19, 39–44}. This activity is independent of the regulatory subunits Hap4 and Hap43^{39–44}. It rather involves cooperative action between the CBC and specific DNA binding transcription factors, such as the GATA factor Gln3 in *S. cerevisiae*⁴². Moreover, CBC has been shown to be a negative regulator of ergosterol biosynthesis and azole resistance in *Aspergillus fumigatus*⁴⁵, while having a positive role on *ERG9* expression in *S. cerevisiae*⁴⁶. Finally, CBC plays an important role in the oxidative stress response of *Aspergillus nidulans* and *Hansenula polymorpha*^{23, 47}. The overexpression of the Hap4B regulatory subunit of *H. polymorpha* (which is orthologous to the HapX proteins⁴⁸) is able to complement the *yap1Δ* sensitivity to hydrogen peroxide in *S. cerevisiae*²³. Our ChIP-seq results also suggest that some of these roles may be conserved in *C. glabrata*. Indeed, the

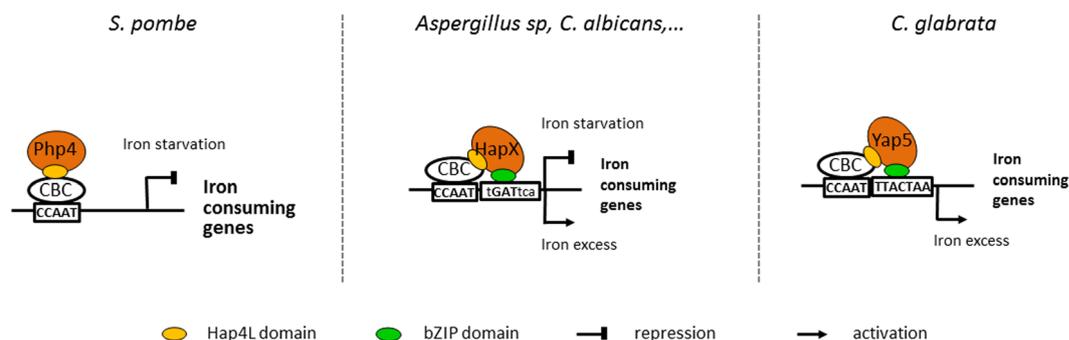


Figure 6. The evolution of the roles of CBC and its regulatory subunits in the control of fungal iron homeostasis. In *Schizosaccharomyces pombe*, Php4 plays an important role in the iron starvation response by repressing the iron consuming genes through its interaction with the CCAAT Binding Complex (CBC) which is mediated by its Hap4Like domain (Hap4L) (reviewed in ref. 15). In *C. glabrata*, Yap5 is a major regulator of the iron stress response which activates iron consuming genes^{21, 29}. Yap5 binding to its targets requires CBC, probably by direct interaction with Hap5 through its vestigial Hap4L domain (this work). However, Yap5 also interacts directly with DNA through its bZIP domain and this interaction is essential for its regulatory activity^{29, 35, 36}. Interestingly enough, the situation in filamentous ascomycetes (e.g. *Aspergillus* or *Fusarium* species) and in *C. albicans* is an intermediate between *S. pombe* and *S. cerevisiae*. HapX plays an important dual role in activating the iron stress response and in repressing the same genes in iron starvation^{11, 12, 18, 20}. HapX interacts with CBC through its conserved Hap4L domain, but it also directly contributes to the binding of the CBC-HapX complex to a bipartite DNA motif, probably through its bZIP sequence^{18, 20, 24, 25}.

glutamate dehydrogenase encoding gene *GDH1* and the ammonium permease encoding gene *MEP3* were targeted by Hap5 in our experiments. Also, a handful of oxidative stress response genes were bound by Hap5 in *C. glabrata* (e.g. *OYE2*, *SOD2*, *CTA1*, ...). Further investigations will be required to decipher the role of Hap5 in these processes.

Methods

Strains and primers. The lists of the strains and primers used in this study are available in Supplementary Files S8 and S9, respectively. All the deleted strains used in this study were obtained from existing collections^{21, 49, 50} and were verified by PCR before use. The genomic myc-tagging of Hap5 was performed as described previously⁵¹. Briefly, myc-tagging cassette was PCR amplified from the M. Longtine's plasmids⁵² with oligonucleotides containing homology sequences flanking the desired genomic insertion points in 5'. At least 10 micrograms of purified PCR product was used to transform HTL cells using a standard yeast transformation protocol²¹. Genotyping of the clones growing on selective media was done by PCR. The correct myc-tagging of Hap5 was verified by sequencing of the gene and western blot²¹.

The genomic TAP-tagging of Hap5 was obtained with the same strategy except that the *TAP-TRP1* cassette was amplified from the pBS1479 plasmid⁵³.

For the mutagenesis of *YAP5*, we started from the pGRB2-YAP5myc plasmid²¹ and used the Quick change site-directed mutagenesis kit from Agilent, following the recommendations of the supplier. The mutant plasmids were controlled by sequencing. After transformation in *C. glabrata* using the "one-step" yeast transformation protocol²¹, the correct expression of the Yap5 mutant proteins was tested by western blot (Supplementary File S6).

Yeast Cultures and Growth Conditions. All cultures were conducted in a rotative shaker at 30 °C. The standard growth media was YPD (Glucose 2%, yeast extract 1%, Bactopectone 1%). For growth in non-fermentable conditions, glucose was replaced by 2% glycerol. The following stress conditions were used: 2 mM iron sulfate in CSM (2% glucose, 0.67% Yeast Nitrogen base, 0.08% Complete Synthetic Media (MP Bio)) for iron excess conditions and 0.5 mM bathophenanthroline disulfonate (BPS) in YPD for iron starvation conditions. The cells were exposed to the corresponding stress for 30 minutes. The strains harboring *HIS3* or *TRP1* selection cassettes were grown in solid or liquid CSM media without the corresponding amino acid.

Chromatin Immunoprecipitation and High-Throughput Sequencing (ChIP-seq). For ChIP, myc-tagged strains were grown in YPD until exponential phase (OD = 0.8). Cross-linking of the cells and ChIP were performed as described previously⁵⁴. The parental HTL (untagged strain) was grown and processed the same way to provide the mock-IP samples. Sequencing of the IPs, Input DNAs and mock IPs samples and primary data analyses (quality controls and mapping of the reads) were performed as described previously⁵⁴. Peak calling was performed with the bpeaks software⁵¹, using both the Input DNA and the mock IP as references. For peak calling using the Input DNA as reference, the bpeaks parameters were T1 = 1.9, T2 = 6, T3 = 1, T4 = 0.7. For peak calling using the Mock IP as reference, the bpeaks parameters were T1 = 1.9, T2 = 6, T3 = 1, and T4 = 0. Only the peaks that were found with both analyses were kept for further processing. These peaks were then manually checked on a genome browser⁵⁵ to discard artefactual peaks (e.g., peaks centered on a tRNA locus, peaks perfectly overlapping a highly expressed ORF) which would have escaped the bpeaks filter. Peaks located outside of a promoter region (i.e. between convergent ORF or inside ORFs) were also discarded from the final list presented in Supplementary File S2.

DNA Motif Enrichment analyses. DNA sequences of ChIP peaks were retrieved from their genomic locations (BED file) using the “getfasta” function from the BEDTOOLS suite⁵⁶. These genomic sequences were used as inputs for the peak-motif tool to search for regulatory motifs⁵⁷. An additional filtering step was added to the standard peak motif procedure to discard low complexity motifs (e.g., CCCCCC).

The co-occurrences of YRE and CCAAT motifs in the ChIP peaks shared by Yap5 and Hap5 were confirmed by visual inspection of the peak sequences using IGV⁵⁵. The genome-wide search of YRE-CCAAT co-occurrences with a spacing of 10–14 bp in *C. glabrata* promoters was performed using the DNA pattern search in RSAT⁵⁸ and a dedicated R-script which automatically identified co-occurrences in promoters and measured the distance between the two motifs.

Network Building. The ChIP peaks were assigned to genes as described previously⁵¹. When a peak was located in a divergent promoter (i.e., an intergenic region in between two divergent genes) the two genes were fused in one target in the network named “gene 1/gene 2”. The network was represented using the igraph R library⁵⁹. The presence of a ChIP peak was used as the parameter to define interactions (arrows) between Hap5 and its targets. The GO criterion was used to differentially color the target promoters (Fig. 1). GO analyses were performed using the “GO term finder” tool at the CGD database, with default parameters⁶⁰.

Transcriptome Analyses. Knock-out and wild type strains were grown in 50 mL of YPD until exponential phase (OD = 0.8) and then stressing agents were added. After 30 min, 15 mL of each cell cultures were flash-frozen in two volumes of cold ethanol and collected by centrifugation. Total RNA was extracted, quality controlled and quantified as described previously²¹. One microgram of total RNA was used for fluorescent cDNA synthesis according to the amino-allyl protocol²¹. The cDNAs were labeled with Cy3 and Cy5 and hybridization was performed as previously described²¹. Two biologically independent experiments were performed for each condition, using dye switch. We used custom *C. glabrata* Agilent arrays in an 8 × 60 k format (array express accession number: A-MEXP-2402). After overnight hybridization and washing, the slides were scanned using a 2-micron Agilent microarray scanner. The images were analyzed using the feature extraction software (Agilent technologies) and normalized using global LOESS⁶¹. The mean of the biological replicates was calculated. A gene was considered as being differentially expressed if its mean absolute Log₂(fold change) value was more than 0.5 and if its expression variation was considered as being statistically significant using the LIMMA package with a cut-off p-value of 0.05⁶².

Real Time Quantitative PCR (Q-PCR) analyses. For RNA extraction, cDNA synthesis and qPCR, cells were grown in the appropriate medium until exponential phase (OD = 0.8), then stressing agents were added for 30 min when required. Cells were then snap-frozen in cold ethanol and collected by centrifugation. Cells lysis was mechanically performed with glass beads using a Fastprep[®]-24 bead beater (MP Biomedicals). Total RNA extraction was carried out using the RNeasy extraction kit (Qiagen) following the manufacturer’s instructions. The concentration of each sample was determined using NanoPhotometer[®] spectrometer (IMPLEN). For each sample, 1 µg of the total RNA were DNase treated using Turbo DNA-free kit (Ambion). After DNase treatment, 0.2 µg of total RNA were used to perform cDNA synthesis using Superscript II Reverse Transcriptase according to the manufacturer’s instructions (Invitrogen). The resulting cDNA were diluted to three different concentrations (1:10, 1:20 and 1:40). Quantitative PCR reactions were performed on a C1000 TM Thermalcycler (Bio-rad) with a 2X SYBR Green master mix (Promega). The qPCR reaction mixture contained 0.5 µM of each primer and 4 µL of one of the three dilutions of the cDNA. These dilutions served as triplicate for each sample. The primers used for qPCR are listed in Supplementary File S7. The relative expression for a given gene was generated by calculating the difference in the abundance between the transcripts of this gene compared to the transcripts of the *ACT1* gene, used as an endogenous reference, based on the Δ Ct method. Finally, the expression values were normalized with the expression of the studied gene in the wild type strain grown in glucose given the arbitrary value 1 (Fig. 3).

For Chromatin-immunoprecipitation followed by qPCR (ChIP-qPCR), three serial dilutions (1:4, 1:8, 1:16) of immunoprecipitated samples were simultaneously processed together with Input samples used for normalization. The primers used for qPCR are listed in Supplementary File S7. The enrichment of the *YHB1* promoter was used as an endogenous control. Q-PCR was performed as described above. The relative enrichment of a specific locus in the immunoprecipitated DNA relatively to the Input DNA and to the *YHB1* promoter enrichment was determined using the $\Delta\Delta$ Ct method.

Co-immunoprecipitation experiments and western blots. Co-immunoprecipitation using Hap5-Protein A as bait was performed as described⁶³ except that we started from 100 mL of cell culture at OD = 0.8 in CSM-His media, that we used Dynabeads[™] PanMouse IgG and that all the IP sample was mixed with 2X Laemmli for western blots. Proteins contained in 15 µl of Input or IP samples were separated on 10% SDS-Polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred to Whatman[®] Protan[®] BA83 nitrocellulose membrane (GE Healthcare). Immunoblotting of Yap5-myc wild type and mutant proteins were performed using 1:10000 mouse IgG Anti-cMyc (Roche) and 1:10000 anti-mouse IgG-HRP (Promega) as primary and secondary antibodies. Detection of the signals was performed using G:BOX Chemi XT4 (Syngene) following incubation with UptiLight[™] HRP blot chemiluminescent ECL substrate (Interchim).

Data availability. The ChIP-seq data can be downloaded from the GEO database (accession number: GSE91371). The complete microarray data are available at Array express database under the accession number: E-MTAB-5348.

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Author Contributions

A.T. performed the ChIP and Q-PCR experiments and the bioinformatics analyses. T.D., M.B. and J.B. constructed the strains. M.B. performed the co-immunoprecipitation experiments. G.L. contributed to the bioinformatics analyses. M.G. contributed to the writing of the text. F.D. conceived the experiments, analyzed the results and wrote the manuscript. All authors reviewed the manuscript.

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5.3 Supplementary results

5.3.1 Introduction

In addition to the previous results, we addressed three more issues in order to complete some unresolved questions of the publication. First, we looked for other evidences showing that both Hap5 and Yap5 binding to DNA through their specific motifs is essential to iron excess response. To that end, we used a reporter gene approach, the set-up of which is described in the next part. In parallel, we tried to evaluate the conservation of the Hap5-Yap5 regulation system across various species belonging to the *Saccharomycetales* clade by looking at the promoter sequences of the orthologues of the Hap5-Yap5 regulon. Finally, we also studied the binding of Hap4, the regulatory subunit of the CCAAT-Binding Complex, and compared its targets with those bound by Hap5 and Yap5.

5.3.2 The YRE and the CCAAT motifs are required to activate the iron excess response

To address the question of the importance of Hap5 and Yap5 binding motifs in iron excess response, we decided to use a LacZ reporter gene under the control of different versions of the *GRX4* promoter. For reasons of ease, we chose to make these constructions on plasmids rather than modify the genomic locus of *GRX4*. We obtained the pZLG plasmid construct from Garcia et al., 2010. This plasmid contains the *Escherichia coli* LacZ sequence under the control of *Saccharomyces cerevisiae* *ZWF1* promoter, an *URA3* marker and *S. cerevisiae* ARS sequence (Garcia et al., 2010). Then, Jawad Merhej used PCR and XhoI/KpnI cloning to add *C. glabrata* ARS sequence and a *HIS3* marker, both coming from pGRB2.1-Myc-His plasmid (Frieman et al., 2002). The resulting plasmid is pZLG-HU-CGSC, abbreviated as pSG. J. Merhej then used PCR and SacII/NotI cloning to add the YHB1 promoter, thus transforming pSG plasmid into pSG-promCgYHB1. This last plasmid was the starting point for the plasmid constructions used in our reporter gene assay.

The goal was to replace *YHB1* promoter by *GRX4* promoter. To reach that goal, Thierry Delaveau amplified genomic DNA from HTL strain (Jacobsen et al., 2010) with the following set of primers : 908-PromCgGRX4-SacII-SmaI-PrF and 909-PromCgGRX4-NotI-PrR (see Appendix X for sequences). After SmaI/NotI digestion of pSG-promCgYHB1, he cloned the *GRX4* promoter in the plasmid with the LacZ reporter gene. The plasmid was then checked by Sanger sequencing and transformed into the HTL strain using the “one-step” yeast transformation protocol (Schiestl et al., 1989; Willins et al., 2002). We also mutated either the Yap5 binding motif (the YRE : TTACTAA) or the Hap5 binding motif (CCAAT)

in *GRX4* promoter using the Quick change site-directed mutagenesis kit from Agilent, following the recommendations of the supplier. These mutant plasmids were then checked by Sanger sequencing and transformed into the HTL strain using the “one-step” yeast transformation protocol.

These three transformed strains (HTL + plasmid *GRX4*-LacZ, HTL + plasmid *GRX4*-mutYRE-LacZ, HTL + plasmid *GRX4*-mutCCAAT-LacZ) were exposed to iron excess (2mM FeSO₄ in CSM-His for 30 min) to create a stress. Cells were harvested and processed to perform qRT-PCR according to the protocol used in Thiébaud et al., 2017.

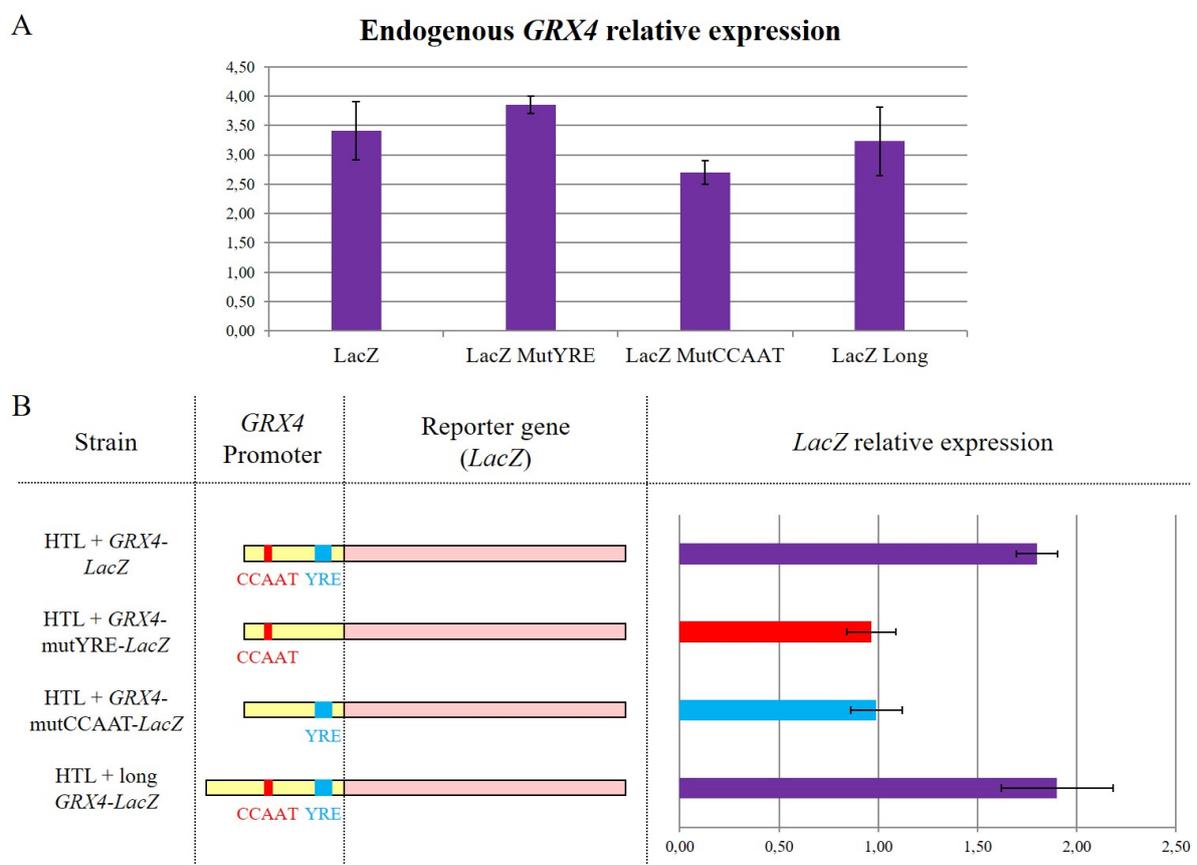


FIGURE 5.1. Analysis of the impact of binding motifs presence on the activation of *GRX4* promoter. Panel A shows the expression of *GRX4* after measurement by q-RT-PCR in HTL + *GRX4*-LacZ, HTL + *GRX4*-mutYRE-LacZ, HTL + *GRX4*-mutCCAAT-LacZ and HTL + long *GRX4*-LacZ strains grown in iron excess (2mM FeSO₄ in CSM-His for 30 min) relative to ACT1 used as an internal control. The experiments were performed two times on biologically independent samples. Error bars thus represent the standard error of the mean. Panel B can be separated in four columns. First column contains the strain names. Second column displays the situation of the promoter (if and how it was mutated). Third column displays the ORF associated to the *GRX4* promoter in second columns. Last column shows the relative expression of LacZ after measurement by q-RT-PCR in HTL + *GRX4*-LacZ, HTL + *GRX4*-mutYRE-LacZ, HTL + *GRX4*-mutCCAAT-LacZ and HTL + long *GRX4*-LacZ strains grown in iron excess (2mM FeSO₄ in CSM-His for 30 min). The values represent the expression levels of LacZ relative to ACT1 used as an internal control. The experiments were performed two times on biologically independent samples. Error bars thus represent the standard error of the mean.

For each sample, the efficiency of the iron excess stress was acknowledged by measuring endogenous *GRX4* expression (Figure 5.1, panel A). With a minimal induction of 2.7, *GRX4* was strongly activated independently of the strain, which means two things : the iron excess stress was efficient and triggered the activation of the iron stress response pathway. It also means that the introduction of plasmids carrying mutations in *GRX4* promoter didn't change the stress response compared to the introduction of a plasmid carrying the wild-type *GRX4* promoter. Figure 5.1, panel B shows the expression of the LacZ reporter gene under the control of *GRX4* promoter during iron excess response. The first thing to notice is that the activation of the reporter gene is weaker than the activation of the endogenous *GRX4* (1.8 vs 3.4). We tried to counter the loss in LacZ activation by increasing the length of the *GRX4* promoter we cloned into the plasmid, in case some unknown regulatory zones were situated further away of the transcription start. It resulted in the strain HTL + plasmid long *GRX4*-LacZ. However, we can see that it didn't impact *GRX4* activation (relative expression of 3.2) nor LacZ activation (relative expression of 1.9), so we didn't try to mutate the Hap5-Yap5 binding sites in this strain. Still, we can clearly see that the activation of LacZ (0.92 vs 1.8) is lost when the promoter is mutated (red and blue columns in the histogram, for the deletion of the YRE and the CCAAT sites respectively), showing that both motifs are necessary to activate *GRX4* following the iron stress. Thus, the absence of Yap5 or Hap5 binding sites in DNA prevents *C. glabrata* from activating the iron excess stress response pathway.

5.3.3 The YRE and the CCAAT motifs are differentially conserved in the *Saccharomycetales*

We wanted to evaluate the conservation of the Hap5-Yap5 regulation system in a variety of pathogenic and non-pathogenic fungi. In order to do that, we studied the conservation of the CCAAT-motif and the YRE (TTACTAA), as well as their spacing. We started by selecting 30 species spanning all the *Saccharomycetales* clade, ranging from *Saccharomyces cerevisiae* to *Yarrowia lipolytica*. We tried to represent all the sub-clades of the *Saccharomycetales* and we favored the species which had reliable genomic sequences available. When several genomes of a same sub-clade were available but their assemblies weren't entirely completed, we selected the genomes (and thus the species) with the smallest number of contigs.

For each of these 30 species, we recovered the promoter sequences of the orthologues of the genes involved in iron excess response in *C. glabrata* (*CCC1*, *GLT1*, *GRX4*, *HEM3*, *ISA1*, *RLI1*, *TYW1*). For

species with properly annotated genomes such as *Saccharomyces* or *Candida* species, the upstream promoter sequences are clearly identified and were easily retrieved in the *Saccharomyces* Genome Database (Cherry et al., 2012; Engel et al., 2014), the *Saccharomyces sensu stricto* website (Scannell et al., 2011) and the *Candida* Genome Database (Skrzypek et al., 2017). More precisely :

- To access the data on SGD, we used the search field to access the gene page, then we went to the “Sequence” tab to use the “Analyze Sequence” tool with the options “S288C vs. other species” and “Fungal Alignment”. From there, we selected the desired species in the “Hits” boxes and picked the “Upstream ORF DNA” option in the sequence type. It allowed to download a fasta file containing the promoter sequences of several *Saccharomyces* species.
- On the *Saccharomyces sensu stricto* website, we went to the “Intergenics” page and we selected the desired promoter sequence in the “Sequence” column. It displays a fasta-like page format that contain the promoter sequence for several species that can be easily saved.
- To recover the data on CGD, we used the search field to access the gene page, then we went to the “Homologs” tab and used the “Genomic +/- 1000 BP (multi-FASTA format)” option to download a fasta files containing the gene sequences and their 1000 flanking bases upstream and downstream for several species.

For the species without properly annotated genomes and unfinished assemblies, we had to use another approach and we defined the promoters as approximately 1000 bases before the transcription start site. Thanks to the SGD, we obtained the *S. cerevisiae* proteic sequences for the orthologues of the seven *C. glabrata* genes mentioned earlier. For the following steps, we primarily used the GRYC (Genome Resources for Yeast Chromosomes) website (<http://gryc.inra.fr/index.php?page=home>) to retrieve the data required to perform our study. On the website, we went to the “BLAST” tab to use the “blastp” tool (Altschul et al., 1990; Altschul et al., 1997). We selected the desired species in the “Select species/strain(s)” option and used the *S. cerevisiae* protein sequences as “Input query”. The other default settings were fitting so we didn’t change them. For the next steps, we only used the blast hits with a s-score greater than 350. The hits allowed us to retrieve the DNA sequence for the orthologues as well as their chromosomal locations. From there, we went to the “Download” tab of the website, selected the species of interest and displayed the fasta files of the scaffold of interest. After locating an orthologue in the associated scaffold, we extracted the promoter sequence of said orthologue. We repeated that for all the orthologues of the 7 genes.

Still, we lacked data for a few species, so we applied the same procedure on the NCBI website which has a bigger database than GRYC : we used the blastp tool to find orthologue proteins and corresponding gene sequences. We used the default settings except for the modification of the “Organism” field to restrain the search and render it faster. We only worked with blast hits with a score greater than 200. We used NCBI Assembly and NCBI Genome to find and extract the promoter sequences of all the remaining orthologues.

Once all the promoter sequences were retrieved, we analysed them to check for CCAAT or YRE presence. When both motifs were found in a promoter, we calculated the distance between them. The results are presented in Figure 5.2, which allows several assessments.

The motifs are conserved in *Candida glabrata* closest species (*Nakaseomyces delphensis* and *Candida bracarensis*) for all genes except *ISAI*. So are the distances (10-14 bases, as mentioned in the previous paper and Figure 5.2) for a majority of genes (*GRX4*, *CCC1*, *TYW1*, *RLI1*, *HEM3* and *GLT1* are also quite close). Another interesting feature is that the system seems to be even more conserved in *Naumovozyma castelli*, where all motifs are conserved as well as most distances, although this species is further from *C. glabrata* than *C. bracarensis* or *N. delphensis*.

The *Saccharomyces* clade is displaying a certain conservation and might even be more homogenous in itself than the *Candidal/Nakaseomyces* clade : motifs and distances are strongly conserved in *GRX4*, *CCC1*, *TYW1* and *HEM3*. Intriguingly, motifs in *ISAI* promoters all sustained a mutation but kept the canonical distance and most likely their role. It was shown in L. Li et al., 2008; L. Li et al., 2011; Pimentel et al., 2012 that *CCC1*, *TYW1* and *GRX4* are bound by Yap5 in *S. cerevisiae* and are still crucial to iron excess response, meaning that at least a part of the regulation is conserved between the species despite the mutation in the sequence.

Last interesting feature relates to the difference of motifs conservation between the promoters : motifs in *GRX4* and *CCC1* are highly conserved across the biggest part of the tree (from *S. cerevisiae* to *Kluyveromyces lactis*) whereas motifs in *GLT1* are lost outside the *Candida/Nakaseomyces* clade. The other genes are an in-between situation, especially *TYW1* and *HEM3* which are strongly conserved in the upper part half of the tree but totally lost in the other half. Distance between the motifs in these genes as well as *CCC1* is also strongly conserved, but the most impressive example resides in *GRX4* : almost everytime both binding motifs are conserved, they are separated by 14 bases. There are only two exceptions, *N. bacillisporus* and *K. lactis*, and even then, the distance is quite close to the canonical

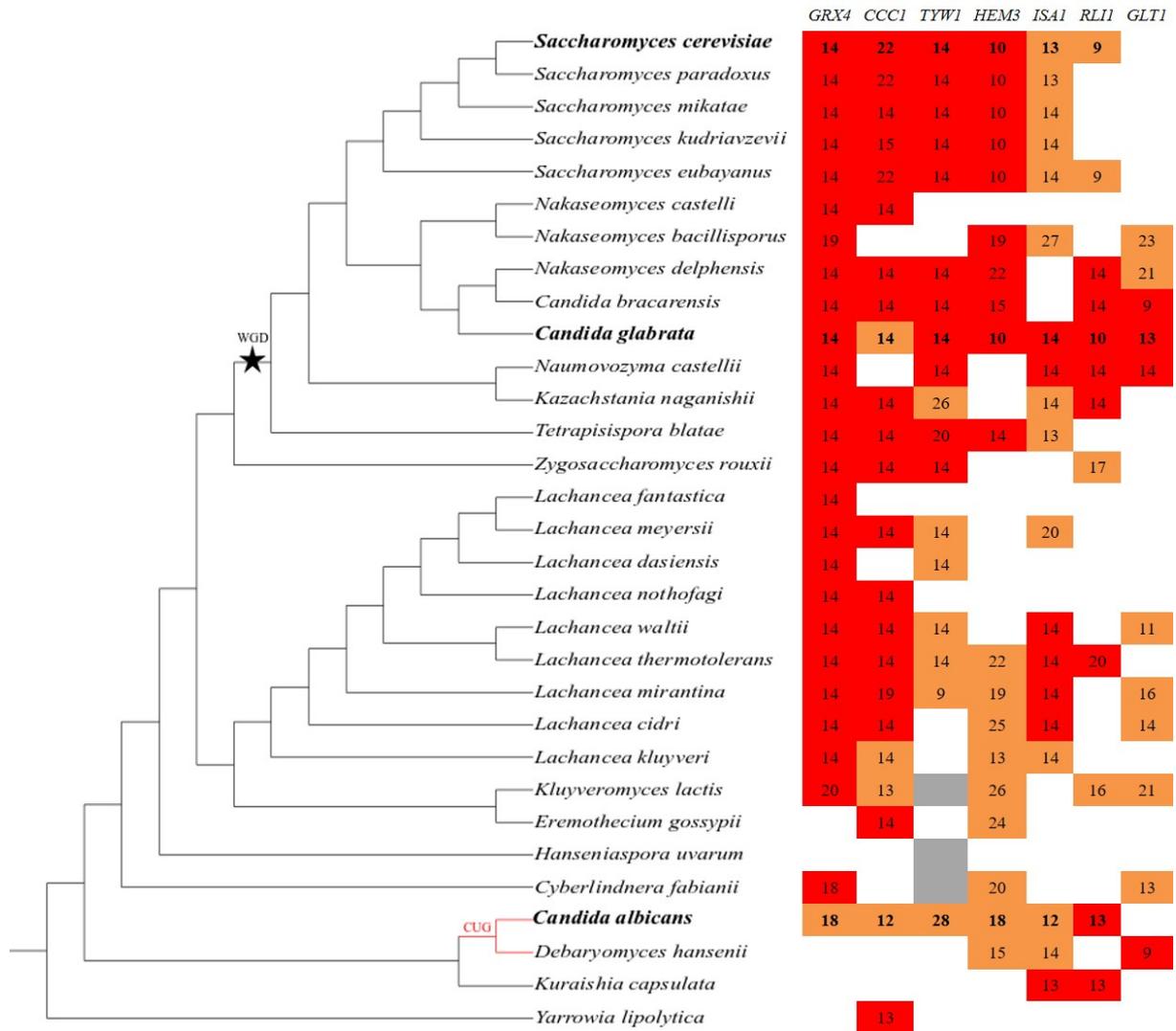


FIGURE 5.2. Analysis of the conservation of CCAAT-motif and YRE and their spacing in 30 species of the *Saccharomycetales* clade. The left panel is a schematic representation of a phylogenetic tree adapted from Aubin Fleiss' data. It is based on the comparison of 350 homologous synteny blocks present in the 30 species considered. The Whole Genome Duplication is indicated by a black star. The CUG-clade is depicted by red branches on the tree. The right panel is a table displaying the results of the analyses of motifs and spacing conservation. Each line represents a species, and each column corresponds to a gene. Color code can be explained as follows : a red cell means both canonical CCAAT-motif and YRE (TTACTAA) were found in the promoter sequence of the corresponding gene and species. An orange cell means both CCAAT-motif and YRE (TTACTAA) were found in the promoter sequence of the corresponding gene and species but one of the motifs has a single mutation. A white cell can have two different interpretations : 1. The motifs have two or more mutations in total. 2. Either only one or no motif was found in the promoter sequence. A grey cell means that no orthologue was found. Finally, the number in the cell is the distance between the two motifs when they are both present ; we only represented distance between 9 and 28 bases. The upper limit was chosen because it corresponds to 5% of the total number of distances (see Fig X). Coincidentally, it is equal to twice the maximum distance found in *C. glabrata*. It is supposed to account for genetic divergence between the species represented in the tree. The bottom limit might account for the steric blockage caused by the binding of two transcription factors : if the binding sites are too close, it is likely one transcription factor will hinder the binding of the other.

distance. The conservation of the distance across such divergent species might even be a stronger sign than the conservation of the motifs that the iron excess response mediated by these genes is conserved.

However, this is only an in silico analysis and it might differ with the regulations that are actually happening in these species. Especially, the presence of one or both motifs doesn't guarantee the binding of the transcription factors on the promoters. Still, CCAAT is a short motif that appears several thousand times in all the upstream sequences of the genome, and this assessment is also true for YRE (TTACTAA), even if it is longer than CCAAT. In *C. glabrata*, over the thousands of likely combinations, 1033 upstream sequences contain a CCAAT/YRE couple. In this case, the average distance between the motifs is 182 bases. Only 27 motifs couples have a distance between 10 and 15 bases, among which 7 are our genes of interest. Then, it would be highly unlikely that both motifs and distances were conserved from *S. cerevisiae* to *K. lactis* without some evolutionary pressure. Confirming this supposition, Merhej et al., 2015 showed that *GRX4* activation by Yap5 in iron excess response is conserved in *Lachancea kluyveri* and *Kluyveromyces lactis*. If we summarise, it means that Yap5 role is conserved in species as far as *K. lactis*, while its binding motif is also conserved and the distance between the CCAAT and the YRE is conserved. This forms a huge body of evidence towards the conservation of the Hap5-Yap5 iron regulation system from *S. cerevisiae* to *K. lactis*.

On another side, variations in the motifs sequence or the distance don't always mean the regulation is lost : distance in *CCCI* promoter in *S. cerevisiae* is bigger than the usual 10-14 bases but the gene kept its functioning and its regulation (L. Li et al., 2008). *CCCI* in *C. glabrata* doesn't contain the exact motifs but it plays a role in iron response (Merhej et al., 2015). But the best example might be found in further species : the Hap43/HapX (the equivalent of *C. glabrata* Yap5 in further species) role seem to be conserved in species such as *Cryptococcus neoformans* (W.H. Jung et al., 2010) or *Aspergillus spp* (Hortschansky et al., 2007; Schrettl et al., 2010; Gsaller et al., 2014).

In the last two articles, they also showed that Afu4g12530 (*CCCI* orthologue in *A. fumigatus*), Afu1g07380 (*GLT1*), Afu4g10690 (*ISA1*), Afu1g10310 (*RLII*), Afu6g12930 (*ACO1*), Afu5g10370 (*SDH2*) expression are up-regulated by HapX in iron excess response, which suggests that the regulation system is conserved, or at least a major part of it (4/7 genes plus *ACO1* and *SDH2* which are targets of Hap5 and Yap5). Interestingly, they didn't report the finding of a YRE motif in these promoters. However, the absence of YRE binding motif can be explained by the fact that the HapX interaction with DNA seems to be degenerated in this species while it is mandatory in *C. glabrata*. Despite the absence of a YRE motif in HapX targets in *Aspergillus spp*, Hortschansky et al., 2015 still reported the presence of a DNA binding motif for HapX, TGAT, which is quite close to a semi-YRE motif. Thus it is likely the regulation system and especially the binding motifs diverged in species further from *C. glabrata* than *K. lactis*, for

example *Cryptococcus spp* or *Aspergillus spp*. However, this system is still partially conserved in these further species, because for example *CCC1*, *GLT1*, *ISA1*, *RLI1*, *ACO1*, *SDH2* are still targeted by HapX during iron stress response.

Interestingly, *Candida albicans* seems to be at a crossroads in the conservation of the system involved in iron excess response. Our data in Figure 5.2 indicates that even if the motifs nor the distances are not perfectly conserved in this species compared to *Candida spp* for example, some slightly mutated motifs are still present and the overall spacing is conserved. This is reinforced by the results of C. Chen et al., 2011 who found that *C. albicans* iron response is partially mediated through Hap43 binding to the same target genes except *GRX4*. Also, the Hap43 DNA binding seems to retain some significance, because Singh et al., 2011 and Srivastav et al., 2018 showed that any mutation in the bZIP domain responsible for the DNA binding of Hap43 impaired its functioning in iron homeostasis. Thus, the system seems to be conserved in *C. albicans*.

Nonetheless, it was reported in Skrahina et al., 2017 that *Candida albicans* Hap43 isn't involved in iron excess response, which is at odds with our suppositions. However, if we look carefully, we can see in these data that *CCC1* activation in iron excess is lost when Hap43 is deleted, which means that Hap43 activates *CCC1* during iron excess response. It resembles a lot Yap5 role in *C. glabrata*. *ACO1*, another up-regulated target of Yap5 in iron excess, shows the same behavior than *CCC1*. In opposition, *HEM3* expression is clearly independent of Hap43 presence in iron excess, which doesn't resemble the *C. glabrata* model. Thus, with these contradictory data on 3 orthologues of Yap5 targets, it would be interesting to look at the expression of the other Yap5-targeted orthologues in *C. albicans* in iron excess. Hap43 could still have a role in iron excess response, but this role would be much more restricted than the role of Yap5 in *C. glabrata* or HapX in *Aspergillus spp*.

Another point we didn't entirely approach is the role of the Hap5-Yap5 system in iron deprivation stress. In *S. cerevisiae*, the response to iron starvation consists in activating Aft1 and Aft2 who in turn activates the iron uptake genes and the *CTH* genes who post-transcriptionally repress the iron consuming genes (Yamaguchi-Iwai et al., 1995; Yamaguchi-Iwai et al., 1996; Blaiseau et al., 2001; Rutherford et al., 2001; Rutherford et al., 2003; Puig et al., 2005). Later, Pujol-Carrion et al., 2006 showed that *GRX4* regulates the nuclear localisation of Aft1 and Pimentel et al., 2012, showed that the deletion of *YAP5* modifies the cellular localization of Aft1, thus changing its abilities to regulate gene expression. So far, that is the only link between the Hap5-Yap5 system and the Aft system during iron deprivation stress

in this species. In *C. glabrata*, Merhej et al., 2016 demonstrated that Yap5 represses *GRX4* during iron deprivation. There was no other report of Yap5 involvement in iron starvation in *C. glabrata*. Gerwien et al., 2016 proved that Aft1 role and its targets (including *CTH2* gene) in *S. cerevisiae* are partially conserved in *C. glabrata*. They also showed that *CTH2* causes the repression of Sef1, which is an activator of iron-consuming genes but reported no role for Yap5. In *C. albicans*, the equivalent of the Hap5-Yap5 system is conserved with the huge role of Hap43 : during iron starvation, Sef1 activates iron uptake genes as well as Hap43 (C. Chen et al., 2011). Hap43 then represses Sfu1 and iron consuming genes (C. Chen et al., 2011; Singh et al., 2011; Hsu et al., 2011) thanks to its interaction with the CCAAT-Binding Complex (Baek et al., 2008). In *Cryptococcus spp* or *Aspergillus spp*, it was shown that HapX has a dual role in iron deprivation and iron excess response and that it controls some sets of genes that are shared between the two opposite conditions (Hortschansky et al., 2007; W.H. Jung et al., 2010; Gsaller et al., 2014). HapX and the CBC have a prominent role in iron deprivation in these species. Given that HapX in *Aspergillus spp* has a role in both conditions, we could think that *C. glabrata* and *C. albicans* had opposite evolutionary directions : Yap5 retained a strong activating role in iron excess and a weak negative role in iron deprivation while Hap43 retained a weak activating role in iron excess and a strong negative role in iron deprivation.

5.3.4 Hap4 might still interact with the CBC during iron excess response

In addition to deciphering the regulation network of Hap5 and Yap5, we also wanted to investigate the network associated to Hap4, the other known regulatory subunit of the CBC. To that end, we performed ChIP-seq experiments to study Hap4 binding on DNA and determine its target genes. After peak-calling procedure, manual verification of the peaks and identification of the target promoters, we found that Hap4 had 314 peaks corresponding to 437 potential targets. 104 of these targets are common with Hap5 targets (Figure 5.3), including the 7 genes of the iron regulon (*CCC1*, *GLT1*, *GRX4*, *HEM3*, *ISA1*, *RLI1*, *TYW1*) and several genes of the respiratory pathway. Despite the qPCR results in the article showing no involvement of Hap4 in iron excess response, this might suggest the contrary, namely Hap4 being involved in iron excess response through the binding of the 7 target genes common to Hap5 and Yap5. However, the binding peaks of Hap4 in the iron regulon genes are much weaker than those of Hap5 (Figure 5.4, panel A). Conversely, Hap4 peaks in the respiratory genes are much stronger than those of Hap5 (Figure 5.4, panel B). This clear difference in peak intensity can be explained by a weaker binding of Hap4 in iron excess response : basically, Hap4 would be the default regulatory subunit of the CBC thus present most of the time around the complex, and it would be replaced by Yap5 during

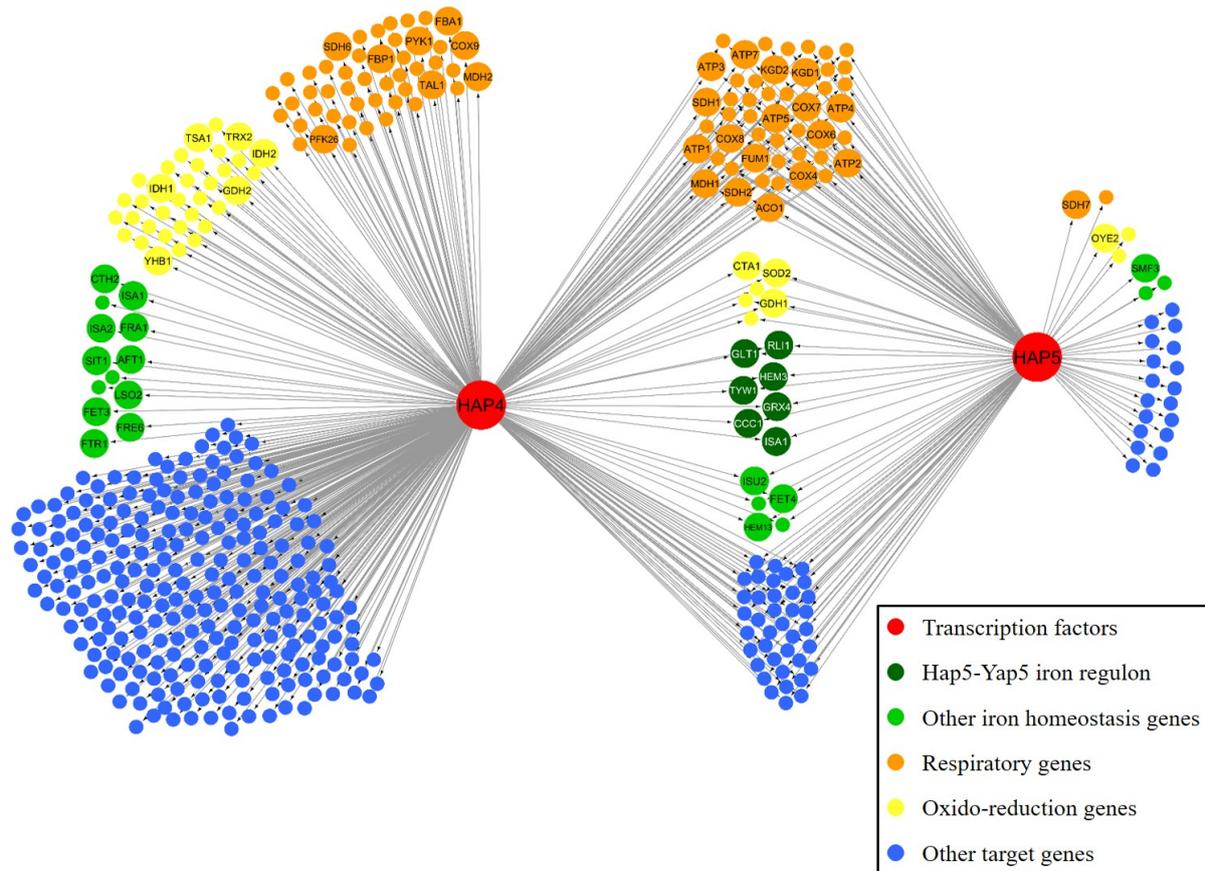


FIGURE 5.3. Representation of Hap4 and Hap5 networks. This network was obtained with Cytoscape (Shannon et al., 2003) using Hap5 ChIP-seq results (Thiébaud et al., 2017) and Hap4 ChIP-seq results (unpublished data). As indicated, red nodes are transcription factors, green nodes are the 7 genes of the Hap5-Yap5 iron excess regulon and blue nodes are all the other targets.

iron excess response. The overall presence of Hap4 around the CBC would be captured in ChIP because of the formaldehyde crosslinking used in the protocol : the crosslinking could lead to the artefactual association of Hap4 to DNA, despite Hap4 not really binding in the promoters of iron regulon genes. Another possibility is that Yap5 interacts with the CBC but does not replace Hap4, which could mean that Hap4 still has a role in iron excess response. This could be tested by measuring the impact of the deletion of Hap4 on gene expression during iron excess.

5.3.5 Conclusion

These supplementary data highlight the fact that the presence of both Hap5 binding motif (CCAAT) and Yap5 binding motif (TTACTAA) is crucial to the activation of the genes involved in the iron excess response. The loss of one of the binding sites is enough to impair the pathway activation. These sites are differentially conserved in the *Saccharomycetales* clade, and so is the distance between them. Nevertheless, it is likely the regulation system spans from *S. cerevisiae* to *K. lactis* even if some divergences

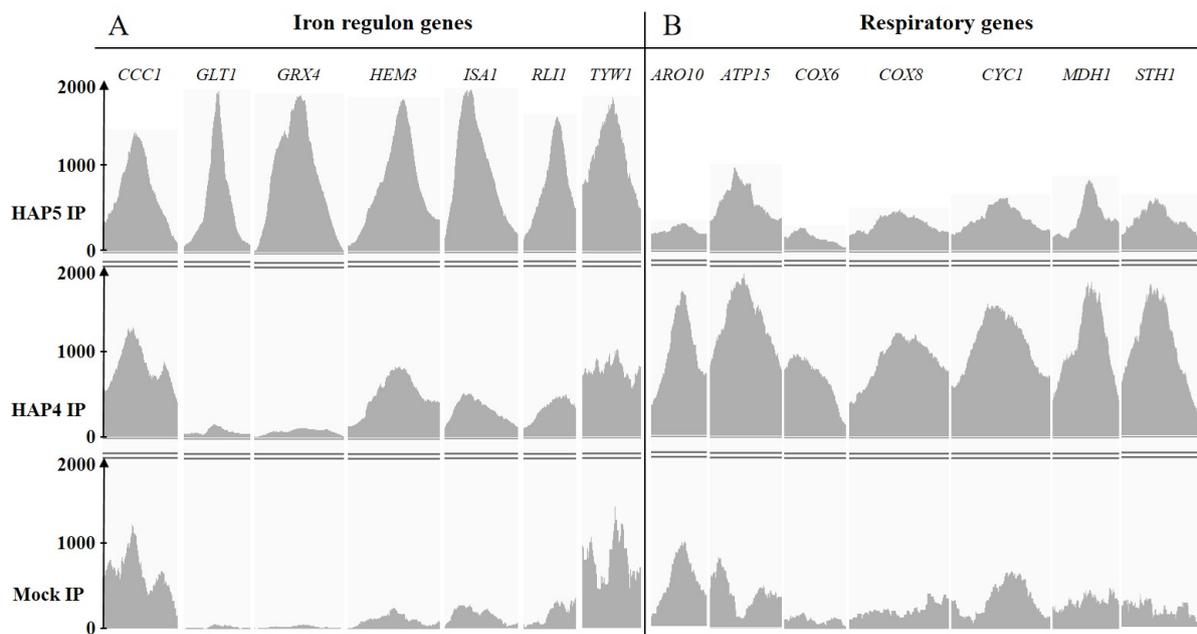


FIGURE 5.4. Representation of the binding peaks of Hap4 in the promoters of 7 iron responsive genes and 7 respiratory genes. This figure was obtained with captures of Integrative Genomics Viewer representation (Robinson et al., 2011; Thorvaldsdóttir et al., 2013). The top lane corresponds to the Hap5 ChIP-seq. The middle lane corresponds to the Hap4 ChIP-seq. The bottom lane corresponds to a mock ChIP-seq. Panel A represents binding in the iron regulon genes. Panel B represents binding in 7 respiratory genes chosen as representatives of the set of targets of Hap4. Scale unit is in number of reads.

are seen in the motif and the distance. This system is also likely conserved in *C. albicans*, and probably derives from the more complete system in *Aspergillus spp.* To be sure, some additional experiments are required, such as ChIP-seq on Hap5 and Yap5 orthologues in iron excess followed by motif enrichment analyses in the ChIP-seq peaks. It would allow to determine their target genes and binding motifs in this condition, as well as the distance between the motifs and clearly decipher the conservation of the system across the species. Another point that would need further clarification is the possible involvement of Hap4 in iron excess response.

Comparative Transcriptomics Reveals New Features of Iron Starvation in *Candida glabrata*

6.1 Introduction

Another transcription factor we took interest in during the Candihub project was the Aft2 transcription factor. Aft2 and its paralog Aft1 are known in *S. cerevisiae* for their role in iron homeostasis, especially during the iron deprivation response. Incidentally, they share several target genes and most of them are involved in iron homeostasis. More precisely, when facing iron depletion, they activate iron uptake genes such as *FET3*, *ATX1*, *SIT1*... and *CTH* genes who post-transcriptionally repress the iron consuming genes (Yamaguchi-Iwai et al., 1995; Yamaguchi-Iwai et al., 1996; Blaiseau et al., 2001; Rutherford et al., 2001; Rutherford et al., 2003; Puig et al., 2005). Aft2 also has some specific targets such as *SMF3* (a vacuolar iron transporter) and *MRS4* (a mitochondrial iron transporter) as shown in Rutherford et al., 2001; Rutherford et al., 2003; Courel et al., 2005. In *C. glabrata*, it was also reported that Aft2 was regulated by Yap1, one of the main factor involved in oxidative stress response (Salin et al., 2008). Gerwien et al., 2016 reported that the deletion of *AFT2* didn't impact cell growth in a wide variety of conditions, including metal restrictions, non-fermentative carbon sources and stress exposure, but except for the previous statements, little was known on Aft2 in *Candida glabrata*. In this frame, the Candihub project was an interesting way to improve our knowledge on Aft2.

We began the study by performing ChIP-seq on Aft2 in iron-depleted conditions. These conditions were reached using BPS. The ChIP-seq allowed us to define the target genes of the factor. The analysis of the ChIP-seq peaks gave 88 target genes. Over 80% of this promoters contain the ACACCC motif which is identical to the consensus identified for Aft2 in *S. cerevisiae* (Courel et al., 2005). A Gene Ontology analysis showed that these target genes are enriched in iron homeostasis and oxidative stress response,

like in *S. cerevisiae*. Obviously, some of these targets are shared with Aft1 or Aft2 in the model yeast. However, we found some genes specific to *C. glabrata*'s Aft2.

We tried to evaluate the impact of Aft2 on the expression of the targets defined previously. We looked at transcriptomic data comparing *aft2Δ* cells to wild type cells in several conditions : YPD (optimal growth), BPS (iron deprivation), selenite (which is also causing an iron deprivation among other things) and cadmium. We noticed almost no significant changes in YPD. However, most of the genes which expression varied in the stress conditions were Aft2 targets in ChIP-seq. One of these common targets is *MAK16b* and its data are quite unsettling.

MAK16b role is unknown while its paralog, *MAK16* is conserved in several species and plays a role in ribosome biogenesis. Interestingly, only *C. glabrata* and some really close species have 2 paralogs of *MAK16*. For example, *S. cerevisiae* has only one *MAK16*. We had at our disposal some transcriptomic data resulting from a time-course study of the stress response of 8 species of yeast (*Saccharomyces cerevisiae*, *Candida glabrata*, *Lachancea kluyveri*, *Lachancea thermotolerans*, *Kluyveromyces lactis*, *Candida albicans*, *Debaryomyces hansenii*, *Y. lipolytica*) to selenite, a pleiotropic stress activating several response pathways including iron starvation and oxidative stress response (Salin et al., 2008). When we looked at *MAK16* expression profile, we saw that it was repressed in the same way in all the species. However, *MAK16b* had the opposite behavior : it was induced during stress.

This led us to look for others Regulatory Outliers (RO) (meaning genes that have different expression profiles when compared to the expression profiles of their orthologs in other species), especially in the Aft2 regulon. To this end, Mariam Sissoko and Hugues Ripoche designed a script, called REGULOUT, to automate the identification of genes with unique expression profiles in their orthogroups, aka to identifying Regulatory Outliers. Briefly, REGULOUT needs two types of file to work : first, a table containing the expression profiles obtained in the different species and second, a table describing the orthology relationships between genes. REGULOUT calculates all the pairwise distances between the expression profiles of all the genes belonging to an orthogroup, then it looks for genes which minimal distance value in the orthogroup is higher than a distance cut-off set up by the user.

When we used REGULOUT on the selenite data previously mentioned, we found 38 ROs in *Candida glabrata*. Among these 38 ROs, we identified 4 ROs specific to iron starvation and bound by Aft2. Additional experiments showed that two of these genes (*DOM34b* and *HBS1*) are required for *C. glabrata* growth in iron depleted conditions. Particularly, the *S. cerevisiae* orthologues of these two genes are

involved in ribosome rescue by the NO GO decay pathway. Thus it might suggest a link between Aft2 response to iron starvation and the No Go Decay pathway in *C. glabrata*.

For this work, I performed the multistress *Candida glabrata* microarray experiments and the subsequent analyses. I performed the transcriptome analyses of the *aft2Δ* mutant strain. I also performed the ChIP experiments, the peak calling and the cis-regulatory motif enrichment analyses.

6.2 Publication



Comparative Transcriptomics Highlights New Features of the Iron Starvation Response in the Human Pathogen *Candida glabrata*

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In this work, we used comparative transcriptomics to identify regulatory outliers (ROs) in the human pathogen *Candida glabrata*. ROs are genes that have very different expression patterns compared to their orthologs in other species. From comparative transcriptome analyses of the response of eight yeast species to toxic doses of selenite, a pleiotropic stress inducer, we identified 38 ROs in *C. glabrata*. Using transcriptome analyses of *C. glabrata* response to five different stresses, we pointed out five ROs which were more particularly responsive to iron starvation, a process which is very important for *C. glabrata* virulence. Global chromatin Immunoprecipitation and gene profiling analyses showed that four of these genes are actually new targets of the iron starvation responsive Aft2 transcription factor in *C. glabrata*. Two of them (*HBS1* and *DOM34b*) are required for *C. glabrata* optimal growth in iron limited conditions. In *S. cerevisiae*, the orthologs of these two genes are involved in ribosome rescue by the NO GO decay (NGD) pathway. Hence, our results suggest a specific contribution of NGD co-factors to the *C. glabrata* adaptation to iron starvation.

Keywords: yeast, Aft, ChIP-seq, NO GO decay, evolution

INTRODUCTION

Candidemia are systemic infections caused by different *Candida* yeast species. They are responsible for high mortality rates (40–50%) in immunocompromised patients, despite the existing treatments (Pfaller and Diekema, 2007). These last 20 years, *Candida glabrata* has become the second leading cause of candidemia, behind the extensively studied *Candida albicans* (Pfaller et al., 2014). Although they have similar names, *C. glabrata* and *C. albicans* are very different species (Brunke and Hube, 2013). *C. glabrata*, in evolutionary terms, is more closely related to the model yeast *Saccharomyces cerevisiae* than to *C. albicans* (Dujon et al., 2004). It actually belongs to the *Nakaseomyces* clade. In contrast to *C. albicans*, *C. glabrata* is a haploid. It is less susceptible to the azole compounds which are commonly used to treat candidemia and can rapidly develop high-level resistance

(Pfaller and Diekema, 2007). Moreover, it has evolved distinct invasive strategies and unique transcriptional responses to stress compared to other pathogenic fungi. For instance, *Candida glabrata* is able to survive and multiply in macrophages by escaping or inhibiting most of the phagolysosome anti-microbial weapons (Kaur et al., 2007; Seider et al., 2011, 2014; Kasper et al., 2015). Identifying the specificities of *C. glabrata* is therefore a key issue to understand its virulence and eventually find efficient treatments.

One obvious way to find *C. glabrata* particularities is comparative genomics (Gabaldon and Carrete, 2015). The comparison of *C. glabrata* and *S. cerevisiae* genomes indicated that *C. glabrata* has lost some genes involved in galactose, phosphate, nitrogen, and sulfur metabolisms (Roetzer et al., 2011). These gene losses resulted in auxotrophy for nicotinic acid, pyridoxine, and thiamine (Dujon et al., 2004; Domergue et al., 2005). These features were hypothesized to be related to the pathogenic nature of *C. glabrata*. However, the sequencing of five more *Nakaseomyces* species, including two species found in human patients and three, non-pathogenic, environmental species, showed that most of these changes were actually shared by both pathogenic and non-pathogenic *Nakaseomyces* species (Gabaldon et al., 2013). This study identified the amplification of the *EPA* genes, which encode for glycosyl-phosphatidylinositol (GPI)-anchored cell wall proteins involved in cell adhesion, stress responses and recognition by the innate immune system (Cormack et al., 1999; De Las Penas et al., 2003, 2015; Domergue et al., 2005; Juarez-Cepeda et al., 2015; Vitenshtein et al., 2016) as the main genomic feature correlating with virulence in this clade (Gabaldon et al., 2013; Gabaldon and Carrete, 2015).

Besides gene gains and losses, phenotypic diversity can also arise from gene regulation divergence (Romero et al., 2012; Roy and Thompson, 2015; Thompson et al., 2015; Johnson, 2017). Hence, numerous cases have been described in which changes in the *cis*- or *trans*- regulatory elements of otherwise conserved genes can lead to the emergence of new functions (Romero et al., 2012; Thompson et al., 2015). For instance, the evolution of pregnancy in mammals was associated with transcriptional network rewiring driven by transposable elements (Lynch et al., 2011, 2015). In yeasts, the loss of an AT rich *cis*-regulatory element in the promoters of oxidative phosphorylation and mitochondrial ribosomal protein genes following a Whole Genome Duplication event (WGD) allowed for the appearance of a respiro-fermentative life style in extent post-WGD species (Ihmels et al., 2005; Habib et al., 2012; Thompson et al., 2013). Comparative transcriptomics (i.e., the comparison of gene expression profiles in different species) has been extensively used in yeasts to identify changes in gene regulation that accompanied the appearance of new physiological properties (Ihmels et al., 2005; Lavoie et al., 2010; Wapinski et al., 2010), to achieve model phylogeny for regulatory evolution (Roy et al., 2013; Thompson et al., 2013) or to predict transcriptional regulatory networks in non-model species (Koch et al., 2017). In the present work, we used comparative transcriptomics to identify regulatory outliers (ROs) in *C. glabrata* and in seven other yeast species. ROs are genes that have very different expression profiles from their orthologs in the other species. To find them, we

designed REGULOUT, a program which automatically identifies genes with unique profiles among their group of orthologs (i.e., orthogroups). We applied REGULOUT to comparative transcriptome analyses of the response of eight yeast species to toxic doses of selenite, a pleiotropic stress inducer. From these data, REGULOUT identified 38 ROs in *C. glabrata*. Using transcriptome analyses of the *C. glabrata* response to five different stresses, we pointed out five *C. glabrata* ROs which were more particularly responsive to iron starvation, a process which is very important for *C. glabrata* virulence (Nevitt and Thiele, 2011; Srivastava et al., 2014). Global chromatin Immunoprecipitation (ChIP-seq) and gene profiling analyses showed that these five genes were under the control of the iron starvation responsive transcription factor Aft2 and that four of them were actually *C. glabrata* specific Aft2 targets as compared to *S. cerevisiae*. Phylogenetic analyses of the promoter sequences of these four genes suggest that their control by Aft2 was fixed after the WGD. Interestingly, the amount of Aft motifs in the promoters of those genes was particularly high in the *Nakaseomyces* sub-clade including the three potentially pathogenic species sequenced to date (namely *C. glabrata*, *Candida bracarensis* and *Candida nivariensis*), as compared with the non-pathogenic *Nakaseomyces* sub-clade or with the *Saccharomyces* genus. Among these four genes, two (*HBS1* and *DOM34b*) were required for optimal growth of *C. glabrata* in iron limited conditions. In *S. cerevisiae*, the orthologs of these two genes are involved in ribosome rescue by the NO GO decay (NGD) pathway. Hence, our results demonstrate the power of comparative functional genomics to identify novel regulatory systems in non-model species and suggest a specific contribution of NGD co-factors to the *C. glabrata* strategy for its adaptation to iron starvation.

MATERIALS AND METHODS

Strains and Growth Conditions

For comparative transcriptomic analyses, we used the following strains: *Saccharomyces cerevisiae* S288C, *Candida glabrata* CBS138, *Lachancea kluyveri* CBS3082, *Lachancea thermotolerans* CBS6340, *Kluyveromyces lactis* CBS2359, *Candida albicans* SC5315, *Debaryomyces hansenii* CBS767, *Yarrowia lipolytica* CLIB122. All strains were grown in rich media at 30°C (YPD: 1% bacto peptone, 1% yeast extract, 2% glucose) on a rotating shaker (150 rpm), except the halophilic yeast *D. hansenii* which was grown in YPD supplemented with 0.5 M NaCl.

For *C. glabrata* mutant strain construction, we used the HTL background (*his3-*, *trp1-*, *leu2-*) (Schwarzmueller et al., 2014). The tagging of *DOM34a*, *DOM34b*, *HBS1*, and *MAK16b* with TAP tag was performed by PCR and homologous recombination as described in Merhej et al. (2015). The tagging cassette was PCR amplified from the pBS1479 plasmid (Puig et al., 2001). The tagging of *AFT2* with a myc epitope was performed exactly as described (Merhej et al., 2015) using the myc-His cassette from the Longtine's collection (Longtine et al., 1998). The *AFT2* deleted strain was obtained from the Schwarzmueller et al. (2014) collection. The *MAK16b* deleted strain was obtained by PCR and homologous recombination as described previously

(Merhej et al., 2015). The *HBS1*, *DOM34a*, and *DOM34b* deletion mutants were obtained by a two steps PCR process using large homologous regions as described previously (Schwarzmueller et al., 2014). For all deletions, we used the *TRP1* deletion cassette from the Longtine's collection (Longtine et al., 1998). For all constructs, the positive clones were selected by growth on CSM-TRP media, except for the *AFT2-MYC* tagged construct which was screened on CSM-HIS media (2% glucose, 0.67% yeast nitrogen base, recommended amounts of CSM-HIS or CSM-TRP from MP Bio). The proper insertion of the cassettes at the targeted genomic loci and the absence of wild type versions of the targeted gene was controlled by PCR. All primers used in this study are available in **Supplementary File S1**.

Multispecies Transcriptome Analyses: Basic Experimental Set Up and RNA Extractions

For each species, we first determined the dose of selenite required for a 100% decrease of the growth rate in exponential phase. The results were: 0.5 mM for *C. albicans*, *D. hansenii*, *K. lactis*, and *L. kluyveri*, 1 mM for *C. glabrata*, 5 mM for *L. thermotolerans*, 10 mM for *S. cerevisiae* and *Y. lipolytica*. Next, we performed kinetic experiments in which the cells were grown in YPD (or YPD + salt for *D. hansenii* as indicated above) at 30°C until they reach an optical density (OD) at 600 nm of 0.6–0.7. Then, we split the cultures in two: one sub-culture received a mock treatment while the second was treated by the indicated amount of selenite (sodium selenite from sigma, stock solution prepared at 0.2 M in water). This step defines time zero. Every 10 min from time 10 to time 80 min, 20 mL of each culture were collected and flash-frozen in 30 mL of cold (−80°C) absolute ethanol. The cells were centrifuged (5 min, 4,000 rpm), washed with cold (4°C) distilled sterilized water, centrifuged again and cell pellets were stored at −80°C. In parallel to sample collection, the OD of the two cultures was measured every 30 min for 3 h to assess the impact of the selenite treatment on the growth rate. For each species, 6–10 independent kinetic series were generated. Only the four series which were the closest to the 100% decrease of growth rate as compared to the mock treated cultures were used for RNA extraction and microarray analyses. RNAs were extracted using the RNeasy kit (Qiagen) following the protocol provided by the supplier. The quality of the RNA extracts was checked on agarose gels prior to reverse transcription.

Multispecies Transcriptome Analyses: Microarray Design, Microarray Experiments, Data Analyses

Agilent 8x60k custom microarrays were designed for each species (array express accession numbers: A-MEXP-2402, A-MEXP-2365, A-MTAB-642 to 647). For probes design, we used the Teolenn software version 2.0.1 (Jourden et al., 2010). The genome sequences and ORFs positions used to create probe sequences were downloaded from the Genolevures website. Only the coding sequences were used for probe design, introns and intergenic regions were not considered. The masked genomes (i.e., without repeated sequences) required for Teolenn were

created using RepeatMasker version open-3.2.8 with the “-pa 4 -species fungi -xsmall” arguments. The main features of the design are summarized in **Supplementary File S2**. There were no probe replicates on the arrays but each ORF was covered by eight different probes in average. For microarray experiments, 1 µg of total RNA was used for fluorescent cDNA synthesis according to the amino-allyl protocol (Merhej et al., 2015). The cDNAs were labeled with Cy3 and Cy5 and hybridization was performed as previously described (Merhej et al., 2015). Four biologically independent experiments were performed for each condition, using dye switch. After overnight hybridization and washing, the slides were scanned using a 2-micron Agilent microarray scanner. The images were analyzed using the feature extraction software (Agilent Technologies) and normalized using global LOESS (Lemoine et al., 2006). The mean of the four biological replicates was calculated. A gene was considered as being induced (repressed) if its mean Log₂(fold change) value was more than 0.75 (less than −0.75) and if its expression variation was considered as being statistically significant using the LIMMA package with a cut-off *p*-value of 0.01 (Ritchie et al., 2015) for at least two consecutive time points. The complete microarray data are available at Array express database under the accession numbers: E-MTAB-7022, E-MTAB-7023, E-MTAB-7044 to 7047, E-MTAB-7049 and E-MTAB-7053. The processed dataset is available in **Supplementary Table T1**.

REGULOUT

REGULOUT is a python program which takes as an input a multispecies expression matrix and the repartition of the genes in orthogroups. There are two parameters which need to be set up by the user: the minimal size of the orthogroups to be used and the Euclidian distance cut-off to be applied. Then REGULOUT calculates, for each orthogroup reaching the defined minimal size, the pairwise distances between the expression profiles of the genes belonging to the orthogroup. In a next step, REGULOUT defines as ROs all the genes which minimal pairwise distance in their orthogroup is equal to or higher than the minimal distance cut-off. As an output, REGULOUT provides a text file with the name of the ROs, their minimal distance in their orthogroup, the ID number and the size of their orthogroup. It also generates automatically PNG files with the expression profiles of the orthogroups in which at least one RO has been identified. REGULOUT can be freely downloaded from www.lcqb.upmc.fr/REGULOUT/, together with a tutorial and the input data sets used in this study. More precisely, the input files used in this article were the expression matrix for all species and the composition of yeast orthogroups taken from the PhylomeDB database (Huerta-Cepas et al., 2014). The distribution of the sizes of the orthogroups is available in **Supplementary File S3**. The minimal size of the orthogroups to be used was set up to 8 to work mostly with genes which were conserved in all the eight yeast species that were considered. The impact of this filter on the number of expression profiles analyzed by REGULOUT is indicated in **Supplementary File S2**. The minimal distance cut-off was set up at 3, which corresponded to the 75 percentile of all the possible pairwise distances in the dataset. From the raw output of REGULOUT (440 genes), we filtered out the

genes which were defined as ROs based on expression variations that were poorly reproducible (p -value > 0.01). Because the Euclidian distance that we used is very sensitive to stochastic variations for genes with a large amplitude in their expression changes, we also removed from the RO list the genes belonging to orthogroups in which all the members had the same directionality in their expression variations (see **Supplementary File S4** for an illustration). The filtered REGULOUT output contained 289 ROs which are listed in **Supplementary Table T2**.

Multi-Stress Transcriptome Analyses in *C. glabrata*

All cultures were conducted in a rotating shaker at 30°C in YPD (glucose 2%, yeast extract 1%, bacto peptone 1%). Stress conditions used were: 1 mM for sodium selenite, 1 M for NaCl, 2 mM for cadmium chloride, 5 mM for iron sulfate or 0.5 mM for bathophenanthroline disulfonate (BPS). Stressed and mock-treated cells were collected as described above, 20 min and 40 min after treatment. RNA extractions, microarray hybridization and data normalization were performed as described above. For the *aft2Δ* versus wild type comparisons, the stress concentrations were the same as above but the cells were collected only 30 min after treatment. The complete microarray data are available at Array express database under the accession numbers: E-MTAB-7023, E-MTAB-7042, and E-MTAB-7043.

Chromatin Immunoprecipitation and High-Throughput Sequencing (ChIP-Seq)

For Aft2 ChIP experiments, myc-tagged strains were grown in YPD until exponential phase (OD = 0.8), they were then treated by 0.5 mM of BPS for 60 min. Cross-linking of the cells and ChIP were performed as described previously (Merhej et al., 2016). The parental HTL (untagged strain) was grown and processed the same way to provide the mock-IP samples. Sequencing of the IPs, Input DNAs and mock IPs samples and primary data analyses (quality controls and mapping of the reads) were performed as described previously (Merhej et al., 2016). Peak calling was performed with the bpeaks software, using both the Input DNA and the mock IP as references (Merhej et al., 2014). For peak calling using the Input DNA as reference, the bpeaks parameters were T1 = 1, T2 = 6, T3 = 1, T4 = 0.7. For peak calling using the Mock IP as reference, the bpeaks parameters were T1 = 1, T2 = 6, T3 = 1, and T4 = 0. Only the peaks that were found in both analyses were kept for further processing. These peaks were then manually checked on a genome browser (Thorvaldsdottir et al., 2013) to discard artifactual peaks (e.g., peaks centered on a tRNA locus, peaks perfectly overlapping a highly expressed ORF) which would have escaped the bpeaks filter. Peaks located outside of a promoter region (i.e., between convergent ORF or inside ORFs) were also discarded from the final list presented in **Supplementary Table T3**. For DNA motif prediction, DNA sequences of ChIP peaks were retrieved from their genomic locations (BED file) using the “getfasta” function from the BEDTOOLS suite (Quinlan and Hall, 2010). These genomic sequences were used as inputs for the peak-motif tool to search for regulatory motifs (Thomas-Chollier et al., 2012). An

additional filtering step was added to the standard peak motif procedure to discard low complexity motifs (e.g., CCCCCC). The list of Aft targets from *S. cerevisiae* was obtained from Yeasttract (Teixeira et al., 2018) and completed by the “regulation” pages of the SGD¹, taking all the documented regulatory interactions using DNA binding or expression evidences and keeping only the targets which were found in relevant growth conditions (i.e., metal stress conditions). The raw ChIP-seq data can be downloaded from the GEO database with the accession number GSE116077.

Bioinformatics Analyses: Gene Ontology, Hierarchical Clustering, Promoter Sequence Analyses

Gene ontology (GO) analyses were performed using the GO term finder tool at the Candida Genome Database² for *C. albicans*, *C. glabrata*, and *D. hansenii* (with the names of the *C. albicans* orthologs), at the Saccharomyces Genome Database for *S. cerevisiae*, the two *Lachancea* species, *K. lactis* and *Y. lipolytica* (using the names of the *S. cerevisiae* orthologs). The hierarchical clustering of **Figure 2** was performed using Mev with Euclidian distance, optimization of gene leaf order and average linkage. For phylogenetic analyses of the promoter sequences, we downloaded the promoter sequences (i.e., 500 base pairs upstream of the ATG) from the Genome Resources for Yeast Chromosomes database (GRYC³), except for the *Saccharomyces* sequences which were taken from the www.saccharomycessensustricto.org website (Scannell et al., 2011) and for the *Kazachstania Africana* sequences which were downloaded from the NCBI website. The sequences used can be found in **Supplementary Files S5–S8**.

Western Blot Analyses

The cells were grown in YPD at 30°C until they reach an OD at 600 nm of 0.6–0.7. Then, we split the cultures in two: one sub-culture received a mock treatment while the second was treated by 0.5 mM BPS. This step defines time zero. At 30, 60, and 90 min, 10 mL of each culture were collected and centrifuged (5 min, 4,000 rpm), washed with cold (4°C) distilled sterilized water, centrifuged again and cell pellets were stored at –80°C. Proteins were separated on 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred to Whatman® Protan® BA83 nitrocellulose membrane (GE Healthcare). Immunoblotting of the protein A-tagged proteins was performed with a rabbit IgG-HRP polyclonal antibody (PAP; code Z0113; Dako), which has a high affinity for Protein A. The membrane was stripped by boiling 30 min in 62.5 mM Tris-HCl pH 6.8, SDS 2% and 4 mM DTT, followed by 10 washes in PBS-Tween (0.1%). Then, immunoblotting of the ribosomal protein Rpl3, used as a loading and transfer control, was performed using 1:10000 rabbit IgG Anti-Rpl3 [gift from M. Garcia: refer to (Delaveau et al., 2016)] and 1:15000 anti-rabbit IgG-HRP

¹www.yeastgenome.org

²www.candidagenome.org

³http://gryc.inra.fr/

(Promega) as primary and secondary antibodies, respectively. Detection of the signals was performed using G:BOX Chemi XT4 (Syngene) following incubation with UptiLight™ HRP blot chemiluminescent ECL substrate (Interchim).

Growth Assays

Growth assays were performed in 96 well plates using a TECAN Sunrise machine. Exponential phase growing cultures of the strains to be tested were diluted to OD = 0.1 in a 50 mL sterile Falcon tube. The 96 well plate was filled with 90 μ L of the cultures. Then, 10 μ L of BPS 0.5 mM stock solution (iron starvation conditions, final concentration 0.05 mM) or 10 μ L of sterile water (mock treatment) were added to each well to reach a final culture volume of 100 μ L. Cell growth at 30°C was followed for 24 h. The slope of the linear part of the $\log(\text{OD}) = f(t)$ curve was extracted and used as the growth rate of each culture in exponential phase. Two technical replicates were made in each plate.

RESULTS

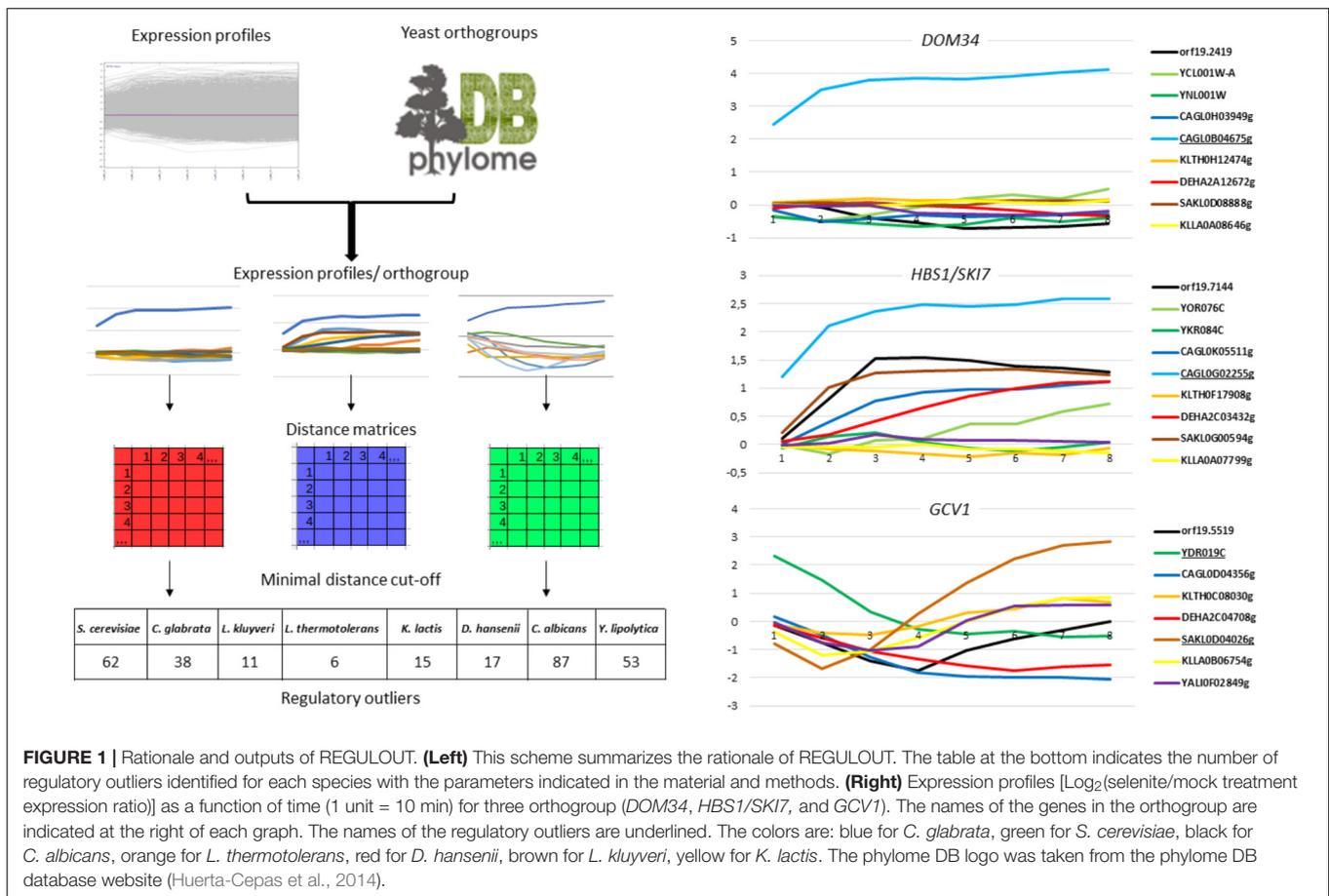
Identifying Regulatory Outliers From Comparative Transcriptomics Data

The starting point of this study consisted in analyzing and comparing the response of eight yeast species (*S. cerevisiae*, *C. glabrata*, *L. kluyveri*, *L. thermotolerans*, *K. lactis*, *C. albicans*, *D. hansenii*, and *Y. lipolytica*) to the stress caused by detrimental doses of selenite. These eight species were chosen because they span the whole Hemiascomycetes tree and because of their high-quality genome annotation at the time when we started the study (Genolevures et al., 2009). Selenite was chosen because it was shown to induce both iron starvation and oxidative stress responses in *S. cerevisiae* (Salin et al., 2008; Herrero and Wellinger, 2015), two stress conditions that *C. glabrata* is facing when invading the human body and being internalized by the cells of the innate immune system (Nevitt and Thiele, 2011; Brunke and Hube, 2013). A key challenge in comparative transcriptomics is to set up the growth conditions to make the physiological state of the different species as similar as possible and to minimize irrelevant differences in gene expression (Kuo et al., 2010; Thompson et al., 2013). To do so, we adjusted the selenite doses used for each species to obtain the same 100% increase in the generation time. Then, RNA samples were collected at eight time points after selenite exposure and compared to the RNAs extracted from a control culture mock-treated for the same duration using microarrays. Four replicates were performed for each species and each time point. **Supplementary Table T1** provides the average \log_2 of the expression ratios between treated and untreated cells for each measured gene in each species for the eight time points, together with a statistical assessment of the significance of the gene expression variations.

As *a posteriori* validation of our approach, we examined the expression patterns of genes from the general Environmental Stress Response (ESR), which were shown to be highly conserved

among the species we studied (Gasch et al., 2000, 2004; Gasch, 2007; Roy et al., 2013; Thompson et al., 2013). We observed that the induction of genes encoding proteasomal subunits and the repression of genes from the Ribosome Biogenesis (Ribi) regulon were consistent between the different species (**Supplementary File S9**). This was a good indication that the eight species were encountering physiologically similar stress conditions. To identify ROs from these 44,723 expression profiles, we designed a program called REGULOUT (**Figure 1**, left panel). REGULOUT takes as input files the expression profiles obtained in the different species and a table describing the orthology relationships between genes (i.e., the names of the genes composing each orthogroup). For each orthogroup taken independently, it calculates all the pairwise distances between the expression profiles of all the genes belonging to the orthogroup. Then, it looks for genes which minimal distance value in the orthogroup is higher than a distance cut-off set up by the user. Those genes with special expression profiles are the so-called “ROs.” The key parameters for REGULOUT are the minimal distance that the user accepts to define a RO and the minimal number of genes for an orthogroup to be considered in the analysis. We applied REGULOUT to our data with parameters corresponding to genes conserved in most of our yeast species (i.e., minimal orthogroup size = 8) and to a minimal distance cut-off equal to the 75 percentile of all the calculated pairwise distances (i.e., $d = 3$). With this criterion, we identified a total of 289 ROs in the eight species, ranging from 6 in *L. thermotolerans* up to 87 in *C. albicans* (**Figure 1** and **Supplementary Table T2**). Careful examination of the corresponding expression profiles led us to distinguish three different situations: directional ROs, quantitative ROs and timing-related ROs. Directional ROs are unique in the directionality of their regulation (i.e., up-regulation, down-regulation or unchanged expression). This is exemplified by the *DOM34* orthogroup in **Figure 1** (right panel). In this example, the expression of one *C. glabrata* member (*CAGL0B04675g*) is strongly induced by selenite while its paralog in *C. glabrata* and its orthologs in the seven other species show unchanged or reduced expression. The quantitative ROs have the same directionality of regulation than other members of the orthogroup, but with much larger amplitude. This is exemplified by the *HBS1/SKI7* orthogroup in the right panel of **Figure 1**. *HBS1* is induced by selenite in *C. albicans*, *D. hansenii*, *L. kluyveri*, and *C. glabrata*, but the induction of the *C. glabrata* gene *CAGL0G02255g* is sufficiently higher than its orthologs to be considered as a RO by REGULOUT. The timing-related ROs have the same directionality and range of regulation than some of their orthologs, but with a different timing. This is exemplified by the *GCV1* orthogroup in **Figure 1** (right panel). *GCV1* is induced in several species following selenite exposure but the pattern of early and transient induction is unique to the *S. cerevisiae* member of this group (*YDR019c*).

Gene ontology analyses indicated that only a few GO terms were enriched in the RO lists of the different species (**Supplementary File S10**). These include genes of the arginine biosynthesis pathway (*ARG3*, *ARG4*, *ARG5,6*, *ARG8*, *CPA1*, *CPA2*), which were much more strongly induced by selenite

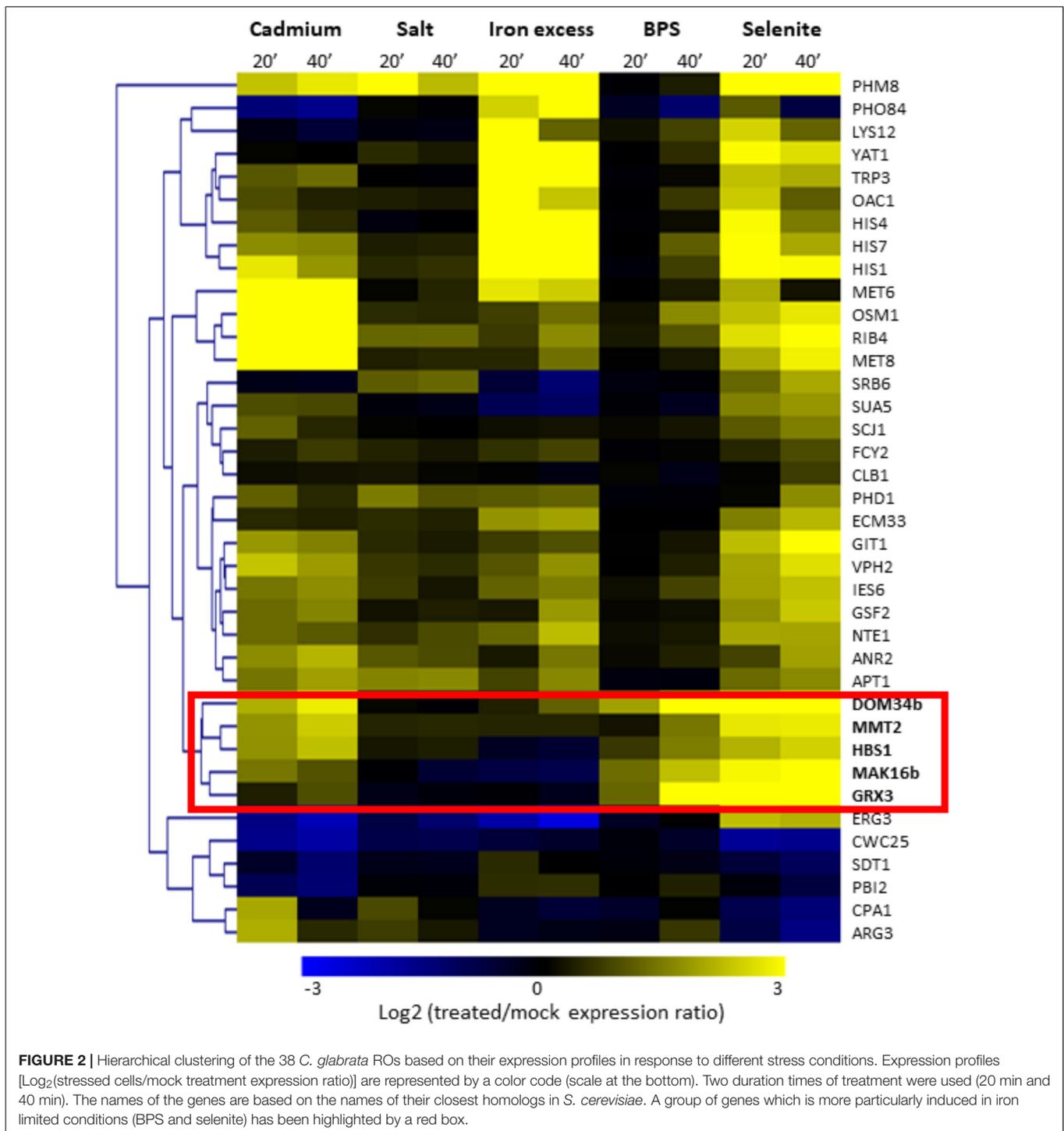


in *C. albicans* than in any other species, and orthologs for the two main actors of a ribosome disassembly pathway named NGD (*DOM34* and *HBS1*), which were specifically induced in *C. glabrata*. Also, several amino-acid metabolism genes (*ARG3*, *HIS1*, *HIS4*, *HIS7*, *CPA1*, *LYS12*, *MET6*, *TRP3*) had special expression patterns in *C. glabrata*. This was already noticed in a previous comparative transcriptomics study analyzing the heat shock stress response in eight yeast species (Roy et al., 2013).

Five ROs in *C. glabrata* Are Induced in Iron Starvation Conditions

As mentioned above, selenite triggers several stress responses (iron starvation, oxidative stress response, DNA damage...) (Pinson et al., 2000; Salin et al., 2008; Perez-Sampietro et al., 2016) and therefore the ROs identified from the selenite comparative transcriptomics data may actually respond to different physiological signals and pathways. We were more particularly interested in identifying ROs in *C. glabrata* which would be linked to the iron starvation response. With this goal in mind, we focused on the 38 *C. glabrata* ROs and took advantage of transcriptome data analyzing the response of *C. glabrata* to five different stress conditions (osmotic stress, iron excess, cadmium, BPS or selenite treatments), which were obtained in the frame of another project (unpublished data). For the present work, we only extracted the data for the

C. glabrata ROs (**Supplementary Table T4**). We then clustered them according to their expression profiles in these five stress conditions (**Figure 2**). Doing so, we could point out a group of five ROs which showed early and strong induction in response to the two iron starvation conditions (namely BPS and selenite treatments) and unchanged or slightly increased expression in response to osmotic and iron excess stresses. This group included the *C. glabrata* orthologs of the *S. cerevisiae* genes encoding the Grx3 glutaredoxin (*CAGL0L11990g*), the Mmt2 mitochondrial iron transporter (*CAGL0E06006g*), the Mak16 ribosome biogenesis factor encoding (*CAGL0F00715g*) and the Dom34 and Hbs1 translation surveillance factors (*CAGL0B04675g* and *CAGL0G02255g*). For the sake of simplicity, we will call the *C. glabrata* genes by the name of their *S. cerevisiae* orthologs in the rest of the article. For *DOM34* and *MAK16*, two paralogs exist in *C. glabrata*. In these cases, the BPS and selenite inducible versions will be called *MAK16b* and *DOM34b* and the versions with unchanged or repressed expression patterns will be called *MAK16a* (*CAGL0G06248g*) and *DOM34a* (*CAGL0H03949g*). Among these five ROs, three were directional ones (*GRX3*, *DOM34b* and *MAK16b*), meaning that they were induced by selenite only in *C. glabrata*, and two were quantitative ROs (*HBS1* and *MMT2*), meaning that they were also induced in other species, but to a lesser extent than in *C. glabrata* (**Supplementary File S11**).



GRX3, DOM34b, HBS1, and MAK16b Are New Targets of the Aft2 Transcription Factor in *C. glabrata*

Interestingly enough, these five genes were also induced by cadmium in *C. glabrata* (Figure 2). In the model yeast *S. cerevisiae*, BPS and selenite trigger an iron starvation transcriptional response which is controlled by the two

paralogous transcription factors Aft1 and Aft2 (Blaiseau et al., 2001; Rutherford et al., 2001, 2003; Courel et al., 2005; Salin et al., 2008; Perez-Sampietro and Herrero, 2014; Perez-Sampietro et al., 2016). However, only the Aft2 regulon is reproducibly induced by cadmium, while most of the Aft1 regulon remains unchanged or is even repressed (Fauchon et al., 2002; Caetano et al., 2015). The expression profiles of the five ROs identified in Figure 2

hence suggested that their particular stress regulation pattern in *C. glabrata* could be due to Aft2.

The Aft2 regulon was not previously deciphered in *C. glabrata*. Then, to test our hypothesis, we conducted genome-wide chromatin immunoprecipitation (ChIP-seq) on a Aft2 myc-tagged strain in iron starvation conditions induced by BPS treatment. ChIP-seq analyses identified 63 promoters bound by Aft2, corresponding to 88 potential gene targets (**Supplementary Table T3** and **Figure 3**, left panel). Sequence analyses of the ChIP peaks identified the ACACCC motif as being the most enriched in the Aft2 bound locus, being present in 80% of the target promoters (**Figure 3**, left panel). This motif is identical to the consensus previously identified for Aft2 in *S. cerevisiae* (Courel et al., 2005; Conde e Silva et al., 2009). GO analyses revealed an enrichment in genes involved in iron homeostasis (p -value = 5.15×10^{-8}) and oxidative stress response (p -value = 0.00276) (**Figure 3**, left panel). More specifically, Aft2 targeted genes belonging to the iron homeostasis category were involved in the reductive iron uptake pathway (*FRE8*, *FET4*), the siderophore iron uptake pathway (*SIT1*), the intracellular iron transport (*MRS4*, *SMF3*, *CCC1*, *MMT2*) and the iron-sulfur cluster biogenesis and assembly pathway (*ISU1*, *ISU2*, *ISD11*, *NFS1*, *YAH1*). In connection with redox homeostasis, Aft2 bound the promoters of the genes encoding the reductases *HBN1*, *OSM1*, *YHB1*, *RNR1*, *RNR2*, and *ERG4*, the superoxide dismutases *SOD1* and *SOD2*, the catalase *CTA1* and the peroxiredoxin *AHP1*. Interestingly, Aft2 also targets genes involved in autophagy (*ATG8*, *ATG19*, and *ATG41*), which may be consistent with the recent finding that mitophagy is triggered by iron starvation in *C. glabrata* (Nagi et al., 2016).

Then we compared the set of targets identified in *C. glabrata* with those of Aft2 and Aft1 in *S. cerevisiae* (**Figure 3**, left panel). Half of the *C. glabrata* Aft2 targets were also targets of Aft1 and/or Aft2 in *S. cerevisiae* (**Figure 3**, left panel). Among these conserved targets is *MMT2*, which was consistent with our observation that *MMT1* and *MMT2* are also induced by selenite in *S. cerevisiae* (**Supplementary File S11**). Reciprocally, 50% of the Aft2 targets were specific to *C. glabrata* ("specific" meaning here as compared to *S. cerevisiae*). Among these *C. glabrata* "specific" Aft2 targets were *GRX3*, *DOM34b*, *HBS1*, and *MAK16b*.

To measure the impact of Aft2 on the expression of its potential targets, we performed transcriptome analyses comparing the *aft2Δ* and wild type strains grown either in optimal conditions (YPD), iron starvation caused by BPS or by selenite, and cadmium exposure. Twenty-four of the 63 promoters bound by Aft2 (38%) were associated to a gene which showed a significant decrease of expression in the *aft2Δ* cells in at least one growth condition. Reciprocally, among the 30 genes which were the most reproducibly affected by *AFT2* deletion, 23 (77%) were directly targeted by Aft2 according to our ChIP-seq results (**Figure 3**, right panel). Among the seven genes which showed changed expression but no binding in ChIP-seq, only one had a potential connection with iron homeostasis (*MET8*, encoding a ferrochelatase) but remarkably six of them (the exception being *GCV1*) had an Aft2 binding motif in the 500 bp upstream of their ATG (data not shown). These differences in expression were observed only in stress conditions

and not in optimal growth conditions (except for *AFT2* itself, which was constitutively deleted in the mutant strain). The four new targets of interest (*GRX3*, *DOM34b*, *HBS1*, and *MAK16b*) showed decreased expression in the *aft2Δ* strain in all three stress conditions, but with different ranges (**Figure 3**, right panel). Hence, our results demonstrate that the increase of expression of *GRX3*, *DOM34b*, *HBS1*, and *MAK16b* in *C. glabrata* is controlled by Aft2.

DOM34b and HBS1 Are Required for Optimal Growth in Iron Starvation Conditions

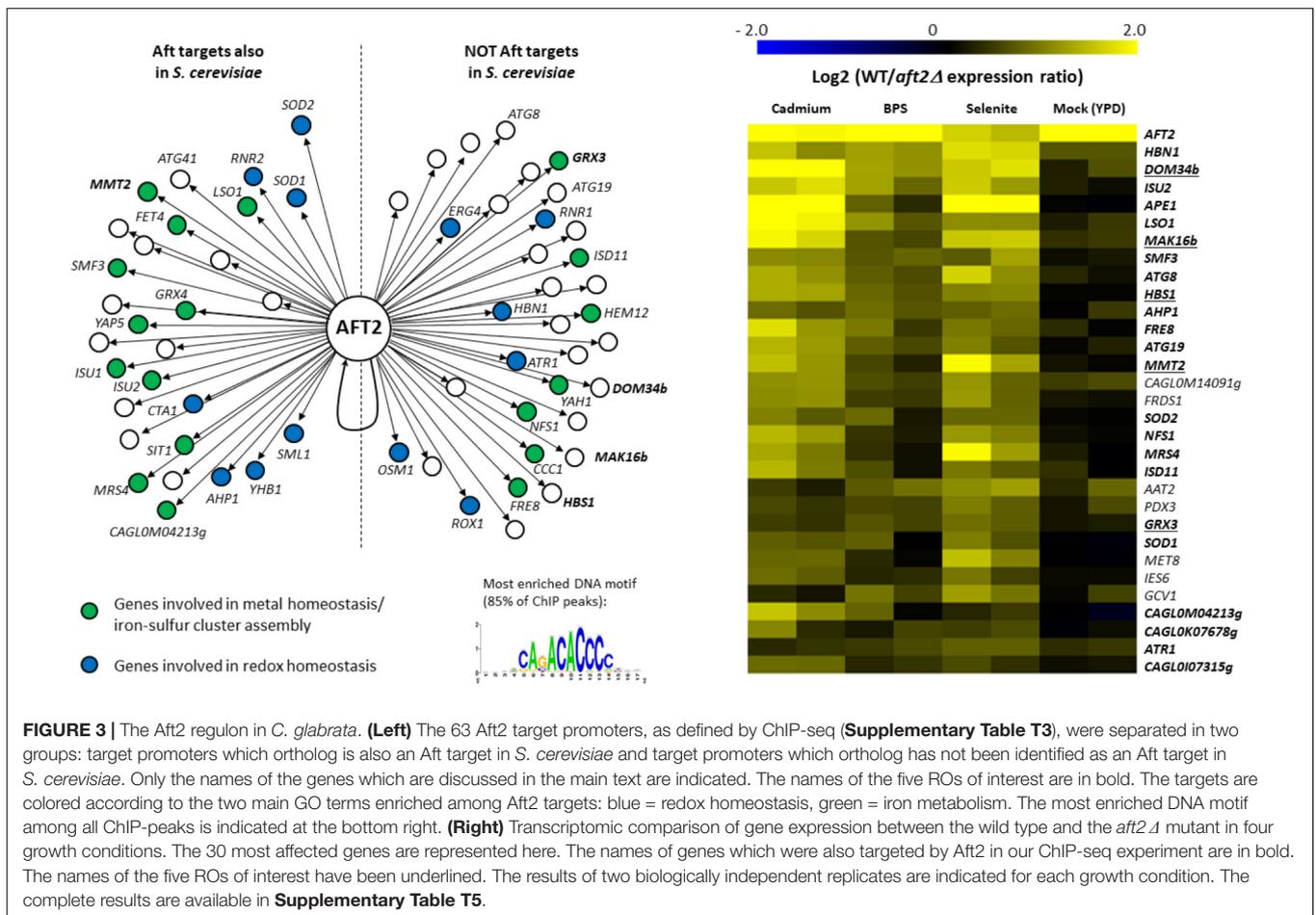
This regulation of *MAK16b*, *DOM34b*, and *HBS1* by Aft2 in *C. glabrata* was particularly intriguing because none of the processes they are contributing to in the model yeast *S. cerevisiae*, namely ribosome biogenesis for Mak16, Dom34 and Hbs1 and ribosome rescue pathway for Dom34 and Hbs1, were directly connected to the transcriptional regulation of iron homeostasis.

Therefore, we assessed the physiological impact of these new regulatory interactions. First, we wanted to know if the increase that we observed at the mRNA level for *DOM34b*, *MAK16b*, and *HBS1* in iron starvation conditions was translated to the protein level. We constructed *C. glabrata* strains with genomic TAP-tagged versions of *DOM34b*, *MAK16b*, and *HBS1*. A strain tagged for *DOM34a* was also constructed as a control for a gene which is not induced by stress at the mRNA level. We performed Western blot analyses on these four strains grown in presence or absence of BPS. We observed a clear and fast induction of Dom34b, Mak16b, and Hbs1 in response to stress, which was perfectly mimicking what was observed at the mRNA level (**Figure 4**, left panel and **Supplementary Files S12, S13**). In contrast, Dom34a displayed unchanged expression levels, as expected.

Second, we assessed the importance of *MAK16b*, *DOM34b*, and *HBS1* in the adaptation to iron starved conditions. We constructed strains deleted for these three genes. We included in our phenotypic analyses strains deleted for *DOM34a* and for *AFT2*. The growth rates of these strains in exponential phase were measured in presence or absence of 0.05 mM BPS and compared to the growth rate of the isogenic wild type strain (**Figure 4**, right panel). Two independent clones were tested for each strain. All mutant strains had similar growth rates compared to the wild type in standard growth conditions (**Figure 4**, upper right panel). In presence of BPS, the *dom34aΔ*, *mak16bΔ* and *aft2Δ* strains did not show any growth defect (**Figure 4**, bottom right panel). In contrast, the *dom34bΔ* and *hbs1Δ* mutants had a decreased fitness in iron starvation conditions.

The Regulation of DOM34b and HBS1 by Iron Starvation Probably Arose From the Whole Genome Duplication (WGD)

As mentioned above, in post-WGD species such as *S. cerevisiae* and *C. glabrata*, there are two Aft transcription factors: Aft2 having a preference for the ACACCC motif and Aft1 being more likely associated to TGCACCC (Courel et al., 2005; Conde e Silva et al., 2009; Goncalves et al., 2014; Srivastava

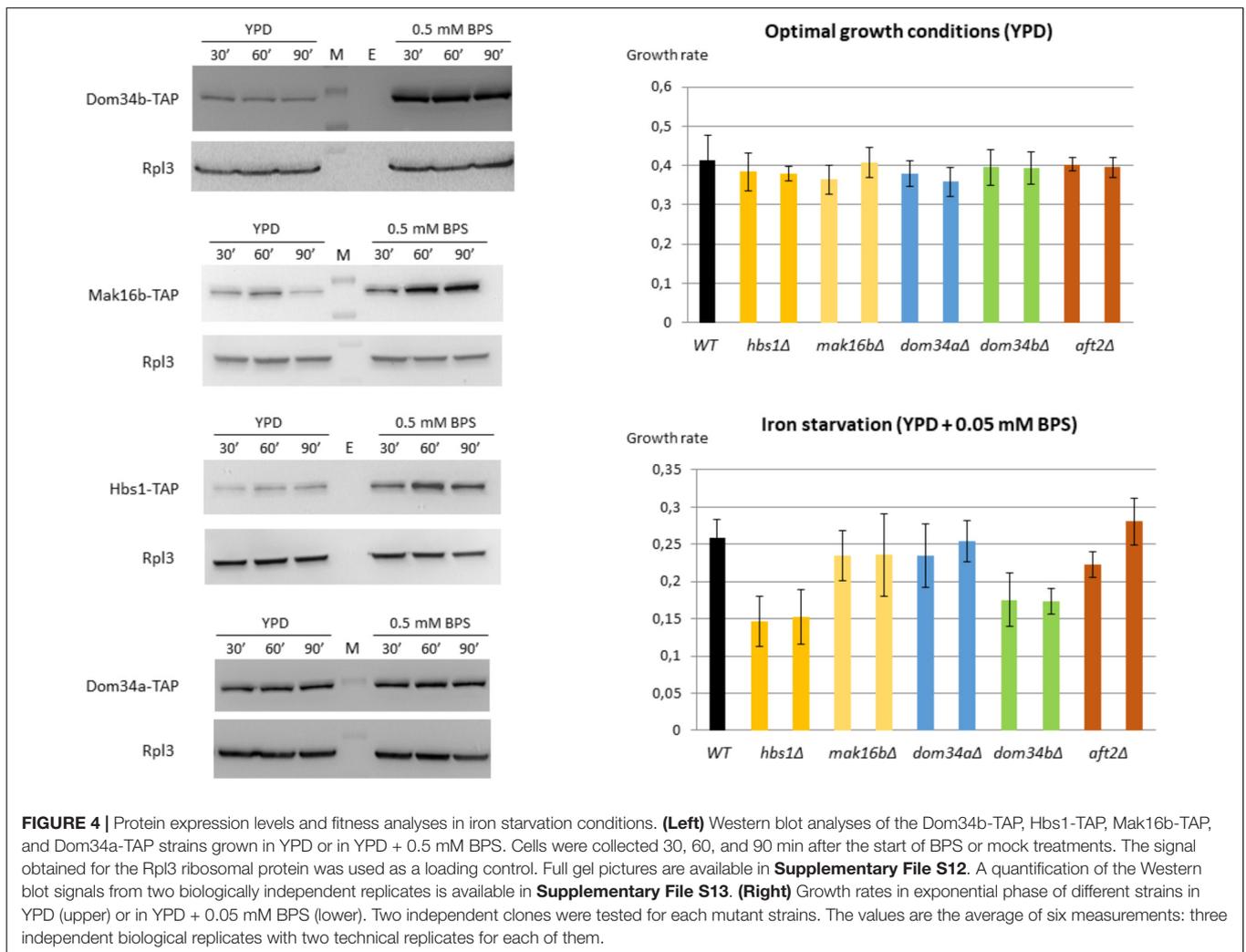


et al., 2015; Gerwien et al., 2016; this work). Besides post-WGD species, the role of the Aft transcription factors in the control of the iron regulon is apparently conserved in the whole *Saccharomycetaceae* family, together with their DNA binding preference for PuGACCC motifs (Conde e Silva et al., 2009; Goncalves et al., 2014). To assess the evolution of the regulation of *DOM34b* and *HBS1* by Aft2 and iron starvation that we characterized in *C. glabrata*, we analyzed the promoter sequences of the orthologs and ohnologs of these two genes in several post- and pre-WGD yeast species spanning the *Saccharomycetaceae* tree, looking for ACACCC (Aft2-like) and GCACCC (Aft1-like) motifs on both DNA strands (**Figure 5** and **Supplementary Files S5, S6, S14**).

In post-WGD species, the evolution of the *DOM34* orthogroup is complex and different situations were observed. In some species (e.g., *Candida glabrata* and its three most related *Nakaseomyces* species, but also *Naumovozyma castellii*, *Kazachstania Africana*, and *Saccharomyces uvarum*) the two ohnologs *DOM34a* and *DOM34b* were retained. In others (e.g., *Tetrapispora blattae*, *Candida castellii*, *Nakaseomyces bacillisporus*) only one copy remained and reciprocal BLAST or synteny analyses could not indicate without ambiguity if it corresponded to the *DOM34a* or *DOM34b* paralog in the other

species. Finally, in most *Saccharomyces* species, one of the two ohnologs was split in two or more ORFs by non-coding insertions (e.g., *YCL001w-a* and *YCL001w-b* in *S. cerevisiae*) and is probably on its way to pseudogenization. Promoter analyses indicated a clear enrichment for Aft motifs in the *DOM34b* lineage of the post-WGD species compared to the *DOM34a* lineage or to the *DOM34* orthologs in the pre-WGD species (**Figure 5**). However, this enrichment is heterogeneous. It is particularly obvious in *C. glabrata* and its close relatives *C. bracarensis*, *N. delphensis*, and *C. nivariensis*, in which the position of one of the Aft2-like motif is particularly well-conserved while the rest of the promoter sequence largely diverged (**Supplementary File S15**). In contrast, there is a loss of the Aft motif enrichment in the *Saccharomyces* species, which seems to be correlated to the pseudogenization of one of the two ohnologs.

A similar pattern was observed for *HBS1*: there is a clear enrichment for Aft motif in the *HBS1* lineage of post-WGD species compared with its *SKI7* ohnolog or with the *SKI7/HBS1* orthologs in pre-WGD species (**Supplementary File S14**). Again, this enrichment in post-WGD species was heterogeneous with, for instance, a clear conservation in the *C. glabrata* sub-clade of the *Nakaseomyces* genus but no Aft motifs in promoters of the *HBS1* versions in the two other *Nakaseomyces* species *C. castellii* and *N. bacillisporus*.

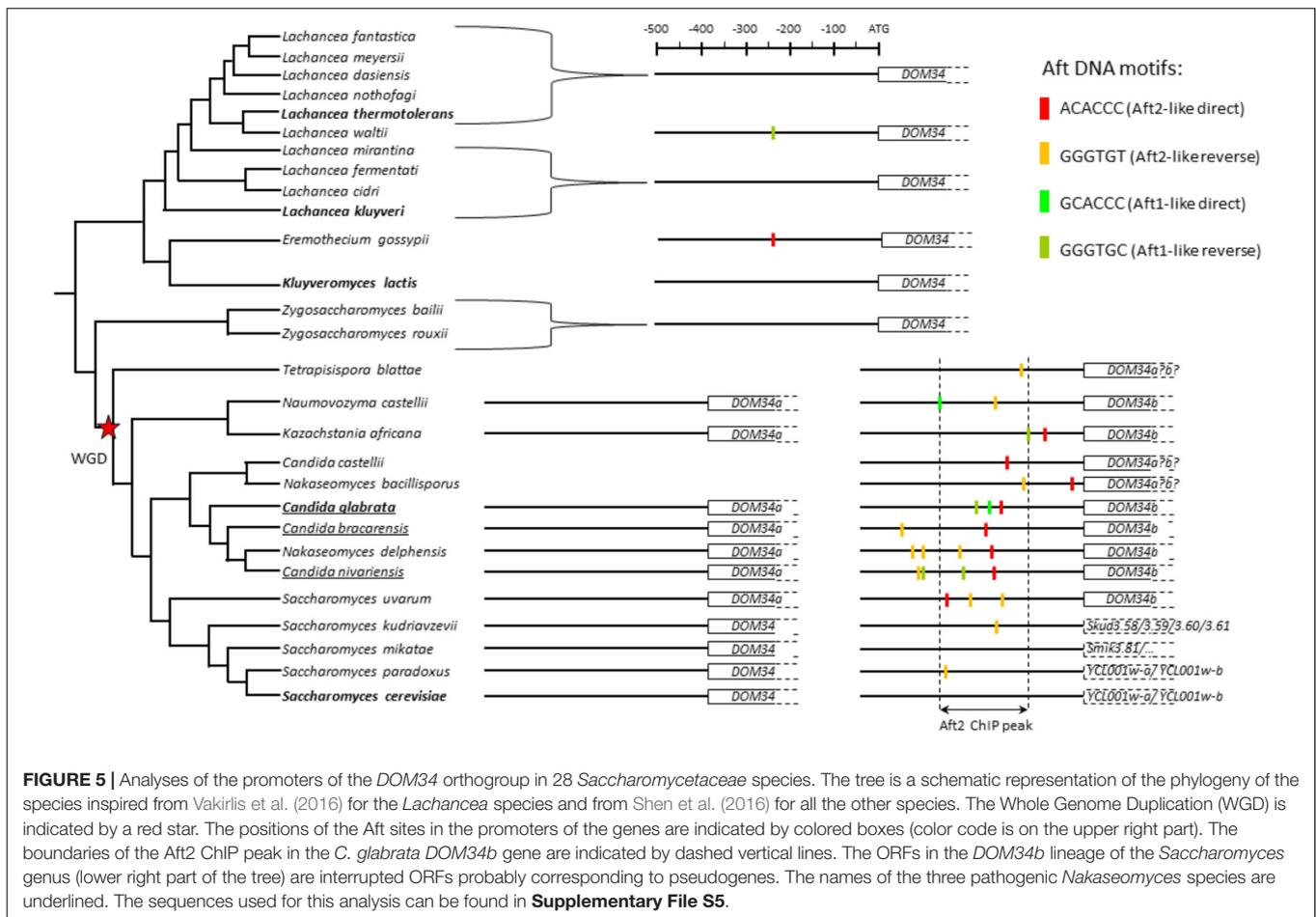


This phylogenetic analysis strongly suggests that the Aft motifs were fixed in the promoters of *DOM34b* and *HBS1* after the whole genome duplication, in a process equivalent to a neo-functionalization event. Then their evolution varied from strong conservation in some species to disappearance in others, possibly reflecting different selection pressures exerted on this new regulation. Yet, it is very important to remember that the presence of an Aft motif does not imply that the motif is active and that the corresponding gene is under Aft regulation. Still, the high conservation of the position of Aft motifs in the promoter of *DOM34b* in *C. glabrata* and its three most related species, while the rest of the promoter diverged, strongly suggests that this motif is under positive selection in these species and then that the regulation that we characterized here for *C. glabrata* is also active in these three species (**Supplementary File S15**).

DISCUSSION

In this study, we designed a program, called REGULOUT, for the identification of conserved genes with divergent

expression profiles from comparative transcriptomics datasets. The inputs of REGULOUT are multispecies gene expression profiles on one hand and the composition of orthogroups on the other. Hence, REGULOUT can be applied to any group of species for which this information is available. The output of REGULOUT is strongly influenced by the distance cut-off chosen and the type of distance used but also by the way the orthogroups were defined and by the stress conditions which were tested. Hence, the label of “ROs” that we introduced in this article is more a technical concept than a straightforward biological feature and REGULOUT should be used as a fast way to sort out lists of potentially interesting cases and not as a tool for gene annotation. We applied REGULOUT to transcriptomics data obtained from eight yeast species responding to the stress caused by detrimental doses of selenite. To assess the power of REGULOUT in highlighting new, biologically meaningful, regulations, we investigated further the 38 ROs identified in the human pathogen *C. glabrata* and we focused on those which were more particularly induced by stress conditions associated to iron starvation.



Iron acquisition is a critical challenge for most microorganisms and a key virulence factor for many fungal pathogens (reviewed in Bairwa et al., 2017; Gerwien et al., 2018). Iron acquisition genes are required for the survival of *C. glabrata* in the macrophages, its adhesion to epithelial cells and for its virulence in animal models (Nevitt and Thiele, 2011; Seider et al., 2014; Srivastava et al., 2014; Brunke et al., 2015). In *S. cerevisiae*, the iron starvation transcriptional response is controlled by the two paralogous transcription factors Aft1 and Aft2. Aft1 activates the expression of genes involved in iron uptake at the plasma membrane (e.g., *FET3*, *FTR1*, *ATX1*, *FRE1-3*, *SIT1*, ...) and genes involved in the cytoplasmic adaptation to low iron conditions, especially the *CTH1* and *CTH2* genes encoding RNA binding proteins involved in the selective degradation of mRNAs encoding iron consuming proteins (Puig et al., 2005, 2008). Aft2 shares some targets with Aft1 but also specifically activates the expression of genes encoding, for instance, the vacuolar iron transporter Smf3 and the mitochondrial iron transporter Mrs4 (Rutherford et al., 2003; Courel et al., 2005; Martinez-Pastor et al., 2017).

Previous studies have shown that many aspects of the iron homeostasis regulation in *C. glabrata* resemble what had been shown in *S. cerevisiae*. Like *S. cerevisiae*, *C. glabrata* strongly depends on the reductive pathway and siderophore uptake for

extracellular iron acquisition (Nevitt and Thiele, 2011; Srivastava et al., 2014; Gerwien et al., 2016). Low affinity iron transport mediated by the Fet4 protein also exists in *C. glabrata* (Srivastava et al., 2014; Gerwien et al., 2016). We showed here that *FET4* is targeted by Aft2 but the sole deletion of *AFT2* had no impact on its induction by BPS, selenite or cadmium (**Supplementary Tables T3, T5**). The key genes of the iron regulon in *S. cerevisiae* are also induced by iron starvation in *C. glabrata* (Srivastava et al., 2015; Gerwien et al., 2016; Nagi et al., 2016). *C. glabrata* Aft1 plays a major role in the up-regulation of membrane iron uptake and is itself induced by iron starvation (Srivastava et al., 2015; Gerwien et al., 2016). Moreover, the enrichment of Cth2 motif in the 3'UTR of genes from the *C. glabrata* iron regulon suggests that the post-transcriptional negative regulation of iron consuming genes is also active in this species (Gerwien et al., 2016). In this work, we showed that the role of Aft2 in the control of intracellular iron trafficking and homeostasis is globally conserved. Especially, the two main specific targets of Aft2 in *S. cerevisiae*, *MRS4* and *SMF3* (Courel et al., 2005), were also regulated by this factor in *C. glabrata* (**Figure 3**). As in *S. cerevisiae* (Blaiseau et al., 2001; Rutherford et al., 2001), the *aft2Δ* mutant exhibited no particular growth defect in response to stress in *C. glabrata* (Srivastava et al., 2014; Gerwien et al., 2016; **Figure 4** of this work).

However, several important differences in iron homeostasis control have also been observed between *C. glabrata* and other model yeast species. For instance, the deletion of the *C. glabrata* ferric reductase encoding genes *FRE8* and *FRE6* does not seem to impact the *C. glabrata* extracellular iron uptake or extracellular ferric reduction activities, in contrast to what has been shown in *S. cerevisiae* and *C. albicans* (Gerwien et al., 2017). Still, these two genes are induced by iron starvation (Nagi et al., 2016; Gerwien et al., 2017) and regulated by Aft2 (this work) and Aft1 (Gerwien et al., 2017), respectively. Moreover, iron starvation was shown to induce the expression of the autophagy genes *ATG32*, *ATG11*, and *ATG8* (Srivastava et al., 2015; Nagi et al., 2016) and to trigger mitophagy in *C. glabrata* but not in *S. cerevisiae* (Nagi et al., 2016; reviewed in Fukuda and Kanki, 2018). We showed here that the iron starvation induction of *ATG8* is under the control of Aft2 (Figure 3). Of note, *ATG32* and *ATG11* are required for *C. glabrata* dissemination in mice and survival in macrophages, respectively (Roetzer et al., 2010; Nagi et al., 2016).

REGULOUT pointed out *GRX3*, *MAK16b*, *DOM34b*, and *HBS1* as being particularly responsive to selenite exposure in *C. glabrata*. Further analyses showed that in *C. glabrata* these four genes were also sensitive to BPS and cadmium treatment, but not to iron excess or osmotic stress (Figure 2). We investigated the regulatory mechanisms underlying these expression patterns and found that the stress response of these four genes is under the control of Aft2 in *C. glabrata* (Figure 3). Our analyses of the evolution of the promoter sequences of the *DOM34* and *HBS1* orthogroups in 14 post-WGD and 14 pre-WGD yeast species revealed enrichment for Aft-like DNA binding motifs in the *DOM34b* and *HBS1* orthologs but neither in the pre-WGD orthologs nor in the *DOM34a* and *SKI7* ohnologs (Figure 5). This strongly suggests that this regulation appeared after the whole genome duplication and was subsequently lost in *Saccharomyces cerevisiae*. This is consistent with previous, more global, observations that duplicated genes are often differentially expressed and evolve divergent regulatory patterns (Gu et al., 2004; Conant and Wolfe, 2006; Tirosh and Barkai, 2007; Wapinski et al., 2007; Thompson et al., 2013), with one of the paralogs retaining the ancestral expression profile while the regulation of the other copy evolves more rapidly (Gu et al., 2005). This can be achieved by changes in the transcription factors that regulate the paralogs (Wapinski et al., 2010; Perez et al., 2014; Pougach et al., 2014), by loss and/or gain of different *cis*-regulatory elements in their promoters (Papp et al., 2003; Zhang et al., 2004; Ihmels et al., 2005) or by a combination of both mechanisms. The latter scenario might be the one at work in our case. Indeed, based on the observation that the Aft transcription factor of the pre-WGD species *K. lactis* fulfills the Aft1 but not the Aft2 functions, it was proposed that the mitochondrial and vacuolar control of iron homeostasis by Aft2 appeared as a neo-functionalization event after the WGD and the duplication of the Aft ancestral gene (Conde e Silva et al., 2009). Then, the acquisition of Aft2 regulation by *DOM34b* and *HBS1* would be concomitant to the emergence of the specific role of this transcription factor. Among the post-WGD species, the enrichment of Aft motifs in the promoters of the *DOM34b* and *HBS1* was especially clear in the *Nakaseomyces* sub-lineage which

contains *C. glabrata* and two other potential human pathogens (*C. bracarensis* and *C. nivariensis*) (Gabaldon et al., 2013) (Figure 5 and Supplementary File S14), as compared to the other *Nakaseomyces* sub-clade or to the *Saccharomyces* genus. A similar pattern was also observed for *GRX3/4*, which showed a dramatic enrichment of Aft motifs in the promoters of the *GRX3* lineage in the *C. glabrata* sub-clade (Supplementary File S16). *C. glabrata*, *C. bracarensis*, *C. nivariensis* and their non-pathogenic relative *N. delphensis* were also the only yeast species to have two *MAK16* orthologs, with the promoter of the *MAK16b* paralog being enriched in Aft DNA motifs (Supplementary File S17). Hence, the presence of two *DOM34*, *GRX3/4* and *MAK16* orthologs and the co-regulation of *DOM34b*, *HBS1*, *GRX3*, and *MAK16b* by Aft2 may be a specificity of this *Nakaseomyces* sub-clade, which is enriched in hosts of the human body (three species out of four). To go further in the characterization of this regulation, it would be interesting to determine the actual contribution of the different Aft motifs in the promoters of the genes by site-directed mutagenesis.

Why were these new regulations fixed in *C. glabrata* and lost in *S. cerevisiae*? In other terms, how could these four genes contribute to iron homeostasis in *C. glabrata*? In *S. cerevisiae*, the cytosolic monothiol glutaredoxins Grx3 and Grx4 play a central role in communicating the mitochondrial iron status to Aft1 and Aft2 (Ojeda et al., 2006; Pujol-Carrion et al., 2006; Kumanovics et al., 2008). In iron replete cells, the mitochondrial Fe-S clusters biogenesis is very active and Fe-S clusters are exported to the cytosol where Grx3 and Grx4 form Fe-S bridged homodimers which has the capacity to transfer its Fe-S cluster to Aft1 and Aft2, therefore decreasing their DNA binding affinity by favoring the formation of Fe/S bridged Aft homodimers (Kumanovics et al., 2008; Ueta et al., 2012; Poor et al., 2014; Chi et al., 2018). This role of Grx3/4 proteins in the regulation of iron homeostasis is conserved from yeasts to humans (Li et al., 2012; Labbe et al., 2013; Jacques et al., 2014; Banci et al., 2015; Encinar del Dedo et al., 2015). It may seem counter-intuitive that *C. glabrata* Aft2 induces the expression of a protein which would negatively control its activity. However, besides its role in the regulation of Aft1/2, Grx3 (and its ohnolog Grx4) also makes important contributions to the oxidative stress response and to the cytosolic and nuclear Fe-S cluster protein assembly (Molina et al., 2004; Herrero et al., 2010; Muhlenhoff et al., 2010; Pujol-Carrion and de la Torre-Ruiz, 2010; Vall-Llaura et al., 2016; Pujol-Carrion and Torre-Ruiz, 2017).

In *S. cerevisiae*, *MAK16* was initially identified in a screen for the maintenance of the killer virus double stranded RNA genome (Wickner and Leibowitz, 1979; Icho et al., 1986). Later on, genetic, biochemical and cryoEM analyses showed that Mak16 is actually involved in the biogenesis of the 60S ribosomal particles (Ohtake and Wickner, 1995; Capowski and Tracy, 2003; Pellett and Tracy, 2006; Altvater et al., 2012; Kater et al., 2017; Zhou et al., 2018). *MAK16* is an essential gene, conserved from yeast to human (Kaback et al., 1984; Wickner et al., 1987). As mentioned above, *C. glabrata* and its three most closely related *Nakaseomyces* species (*C. nivariensis*, *N. delphensis* and *C. bracarensis*) have two *MAK16* paralogs, which we named here *MAK16a* and *MAK16b*. *MAK16b* was obviously not essential

in *C. glabrata* since the *mak16bΔ* mutant cells grew at wild type rates in YPD (Figure 4). As all members of the ribosome biogenesis (RiBi) regulon, *MAK16* was repressed by stress in all the species we examined (Supplementary File S11). This was also true for *MAK16a* in *Candida glabrata*, which was consistently repressed by selenite and by all the other stresses that we tested (Supplementary File S11, unpublished data). Then, the dramatic induction of *MAK16b* by selenite, cadmium and BPS, and its regulation by Aft2, is particularly intriguing. The sole link that can be made between Mak16 proteins and stress responses based on the literature is the fact that they were proposed to form an atypical class of glutathione-S transferases (GSTs) (McGoldrick et al., 2005). GSTs are enzymes which assist the cell in the defense against reactive oxygen species (reviewed in Kalinina et al., 2014). Moreover, glutathione plays a key role in iron sensing by Grx3/4 and in iron-sulfur cluster assembly (Muhlenhoff et al., 2010; Kumar et al., 2011; Ueta et al., 2012; Martinez-Pastor et al., 2017; Cardenas-Rodriguez et al., 2018). Still, this annotation of *MAK16* orthologs as GSTs only relies on immunological criteria, i.e., the Mak16 protein of the trematode *Schistosoma mansoni* was probed by an anti-serum against purified *S. mansoni* GSTs (Milhon et al., 2000), and an actual GST activity was never proven for these proteins. Moreover, the fact that the deletion of *MAK16b* does not alter the growth of *C. glabrata* in iron starvation conditions makes its actual contribution to the iron homeostasis in this species questionable.

In contrast to *MAK16b*, *DOM34b*, and *HBS1* were required for optimal growth in iron starvation conditions, suggesting a role for these genes in the adaptation to iron limitation

in *C. glabrata* (Figure 4). Dom34 and Hbs1 are also highly conserved proteins which are found in all eukaryotes, but they are not essential in yeasts. In *S. cerevisiae*, Dom34 and Hbs1, together with the general translation termination factor Rli1, were initially described as being responsible for a translation surveillance pathway called NGD, which rescues and recycles the ribosomes that stall before translation is completed (Doma and Parker, 2006; Passos et al., 2009; Simms et al., 2014; see Buskirk and Green, 2017; Simms et al., 2017, for recent reviews). Later on, the NGD co-factors were shown to be involved in ribosome biogenesis (Kispal et al., 2005; Yarunin et al., 2005; Cole et al., 2009; Lebaron et al., 2012; Strunk et al., 2012) and in the re-activation of hibernating ribosomes prior to quiescence exit (van den Elzen et al., 2014). Dom34 and Hbs1 also have a role in preventing ectopic translation of mRNA 3' untranslated regions (Guydosh and Green, 2014), that is especially important in conditions in which the activity of Rli1 in regular translation termination is impaired (Young et al., 2015). In *S. cerevisiae*, the expression of *DOM34* and *HBS1* is not regulated by stress and these proteins have not been connected to the regulation of iron homeostasis. So, what could be the functional meaning of the strong induction of *DOM34b* and *HBS1* by BPS, selenite and cadmium in *C. glabrata*? Actually, a quite direct connection can be made between iron starvation and the Dom34-Hbs1 co-factor Rli1. Indeed, Rli1 is an essential Fe-S cluster containing protein (Paul et al., 2015). This iron-sulfur cluster is required for most of Rli1 functions and is very sensitive to redox stress (Kispal et al., 2005; Yarunin et al., 2005; Alhebshi et al., 2012). As a consequence, the number of unrecycled ribosomes in 3'UTRs

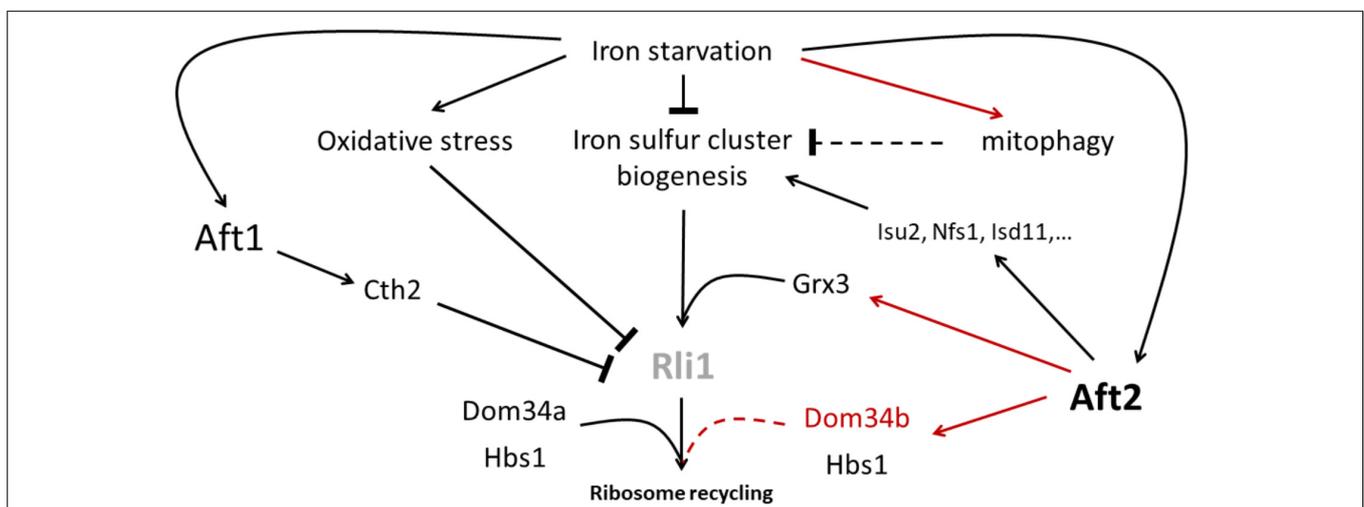


FIGURE 6 | Hypothetical working model for the role of Dom34b and Hbs1 in iron starvation adaptation of *C. glabrata*. Iron starvation alters the functioning of the essential iron-sulfur cluster containing Rli1 in ribosome recycling at many levels: 1- by reducing the iron-sulfur cluster biogenesis potential, 2- by generating oxidative stress which was shown to strongly alter Rli1 activity, 3- by inducing the expression of the Cth2 RNA binding protein which targets part of the Rli1 mRNAs for degradation and 4- specifically in *C. glabrata*, by inducing mitophagy, which may reduce the mitochondrial biogenesis of iron-sulfur clusters. However, iron starvation also triggers the activation of Aft2 which may compensate the decreased activity of Rli1 by overexpressing 1- components of the mitochondrial core iron-sulfur cluster biogenesis machinery; 2- the glutaredoxin Grx3 which is involved in the delivery of iron-sulfur clusters to Rli1 in the cytosol and 3- the Dom34b and Hbs1 ribosome rescue factors, which, together with the constitutively expressed paralog Dom34a, may contribute to increase the ribosome recycling activity of Rli1. In this scheme, the red lines indicate the regulations which are specific to *C. glabrata* as compared to *S. cerevisiae*. The dashed lines indicate hypothetical activities which have not yet been supported by experimental data (namely: an impact of mitophagy on iron-sulfur cluster biogenesis and a role of Dom34b and Dom34a in ribosome rescue in *C. glabrata*).

increases upon treatment with oxidizing agents, such as diamide, which targets iron–sulfur cluster proteins (Guydosh and Green, 2014). Some authors even called Rli1 the “Achilles’ heel” of the cells in oxidizing conditions and hypothesized that Rli1 dysfunction is the main cause of cell death in acute oxidative stress (Alhebshi et al., 2012).

Hence, iron starvation is likely to alter the essential Rli1 activity in ribosome rescue at many levels (Figure 6). Indeed, iron starvation has been shown to render yeast cells more sensitive to oxidative stress (Blaiseau et al., 2001; Matsuo et al., 2017). Moreover, the Fe–S cluster biogenesis, which takes place in the mitochondria, obviously depends on the iron supply (reviewed in Outten and Albetel, 2013). Finally, in *S. cerevisiae* and in *C. glabrata*, iron starvation provokes the Aft1-mediated overexpression of the RNA binding protein Cth2 which targets the Rli1 mRNA to degradation pathways (Puig et al., 2001, 2005; Gerwien et al., 2016). These effects may be particularly and specifically amplified in *C. glabrata* due to the active mitophagy triggered by iron limitation conditions (Nagi et al., 2016), which may also alter mitochondrial Fe–S cluster biogenesis. Thus, it is tempting to hypothesize that the transcriptional induction of *DOM34b* and *HBS1* would be a *C. glabrata* strategy to better cope with the decreased Rli1 activity caused by iron starvation (Figure 6). Interestingly enough, such a transcriptional up-regulation of *DOM34* and *HBS1* to compensate Rli1’s defects has already been described in human erythroblasts (Mills et al., 2016). When differentiating in red blood cells, erythroblasts face a difficult challenge: they completely get rid of their mitochondria and hence lose the capacity of producing iron–sulfur clusters, while having to maintain an active translation of hemoglobin, and therefore an active ribosome rescue process, in the last differentiation stages. They do so by transiently overexpressing Pelota (the human equivalent for *DOM34*) and HBS1L (the human ortholog of *HBS1*), which compensates for the progressive loss of active Rli1 (ABCE1 in Human) (Mills et al., 2016). Then, the hypothetical model that we propose for *C. glabrata* (Figure 6) would be analog to what was described for human erythroblasts. Of note, the induction of *GRX3* by Aft2 can also take place in this model, because Grx3 is involved in the transfer of the Fe–S cluster for the biogenesis of cytosolic and nuclear Fe–S proteins, such as Rli1 (Muhlenhoff et al., 2010) (Figure 6). This model is also consistent with our observation that in *C. glabrata* Aft2 targets several components of the core iron–sulfur cluster machinery. Obviously, many experiments, which go far beyond the scope of this study, will be required to test this working model. Especially, the actual contribution of Hbs1, Dom34a and Dom34b to ribosome rescue in *C. glabrata*, the precise role of

Grx3 in the *C. glabrata* iron starvation response and the potential link between the regulation of these genes by Aft2 and the iron starvation-triggered mitophagy in *C. glabrata* will need to be thoroughly investigated.

AUTHOR CONTRIBUTIONS

MS, HR, and EC made the REGULOUT script and identified the ROs. LJ and SLC designed the microarrays. GeL, SB, and FD performed the multispecies microarray experiments. GaL, AT, and FD analyzed the microarray experiments. MB, LF, and TD built the strains and performed the Western blot analyzes. MB and LF made the growth assays in 96-well plates. AT and JM performed the multistress *C. glabrata* microarrays experiments. MB and AT performed the transcriptome analyses of the *aft2D* mutant strain. AT performed the ChIP experiments, the peak calling and the *cis*-regulatory motif enrichment analyzes. FD did the phylogenetic analyzes of the promoters, conceived the whole project, interpreted the results and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2018.02689/full#supplementary-material>

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6.3 Supplementary results

6.3.1 Introduction

Additionally to these results, we focused on two other questions related to the role of AFT factors and *GRX* genes regulation in iron-starvation response. We studied the binding of Aft2 paralogue, Aft1, in iron-limited conditions and we compared these findings with the transcriptomic impact of *AFT1* deletion performed by other teams. We also tried to understand the differences in *GRX3* and *GRX4* regulation : both genes are involved in iron deprivation response, however it seems that these genes have evolved quite distinct regulation pathways, and even if they are paralogues, they seem to be involved in different networks and have different roles.

6.3.2 Aft1 network in *Candida glabrata*

In addition of studying Aft2 role during iron-deprivation response in *C. glabrata*, we were also interested in understanding a part of the regulation network associated to its paralogue Aft1. Thus, we performed ChIP-seq experiments on myc-tagged Aft1 factor in iron-limiting conditions. To achieve these conditions, we used 0.5 mM of BPS for 30 min. After peak-calling procedure, manual verification of the peaks and identification of the target promoters, we found 109 binding peaks, which correspond to 148 potential targets (Figure 6.1). GO analysis of these targets showed an enrichment in genes involved in homeostasis of transition metals homeostasis ($p = 4.25 \cdot 10^{-9}$) and especially iron homeostasis ($p = 3.52 \cdot 10^{-8}$) with genes in high-affinity reductive iron uptake, siderophore uptake and Fe–S cluster assembly pathways. This was the first time Aft1 role was studied by ChIP-seq in *C. glabrata*. However, several teams had already performed transcriptional studies of Aft1 and other genes potentially involved in iron homeostasis in the last few years.

If we exclude Aft1 and Aft2, Srivastava et al., 2014 showed the involvement of 12 genes in iron homeostasis. These genes are *FTR1*, *FET3*, *CCC2*, *FRE6*, *SIT1*, *FET4*, *FTH1*, *HMX1*, *FET5*, *YFH1*, *CCW14* and *MAM3*. Over these 12 genes, the first 8 are directly bound by Aft1 (Figure 6.1). Additionally, Aft1 also binds *ISU1*, which is known to interact with *YFH1* in *S. cerevisiae* (Gerber et al., 2003; Ramazzotti et al., 2004) and it binds other members of the *FET* and *CCW* families, even if we didn't find *FET5* and *CCW14* among its targets. This shows a huge overlap between iron homeostasis genes defined by Srivastava et al., 2014 and our ChIP results. Later, Gerwien et al., 2016 confirmed the crucial role of Aft1 in iron uptake during iron deprivation in *C. glabrata* and acknowledged that the following

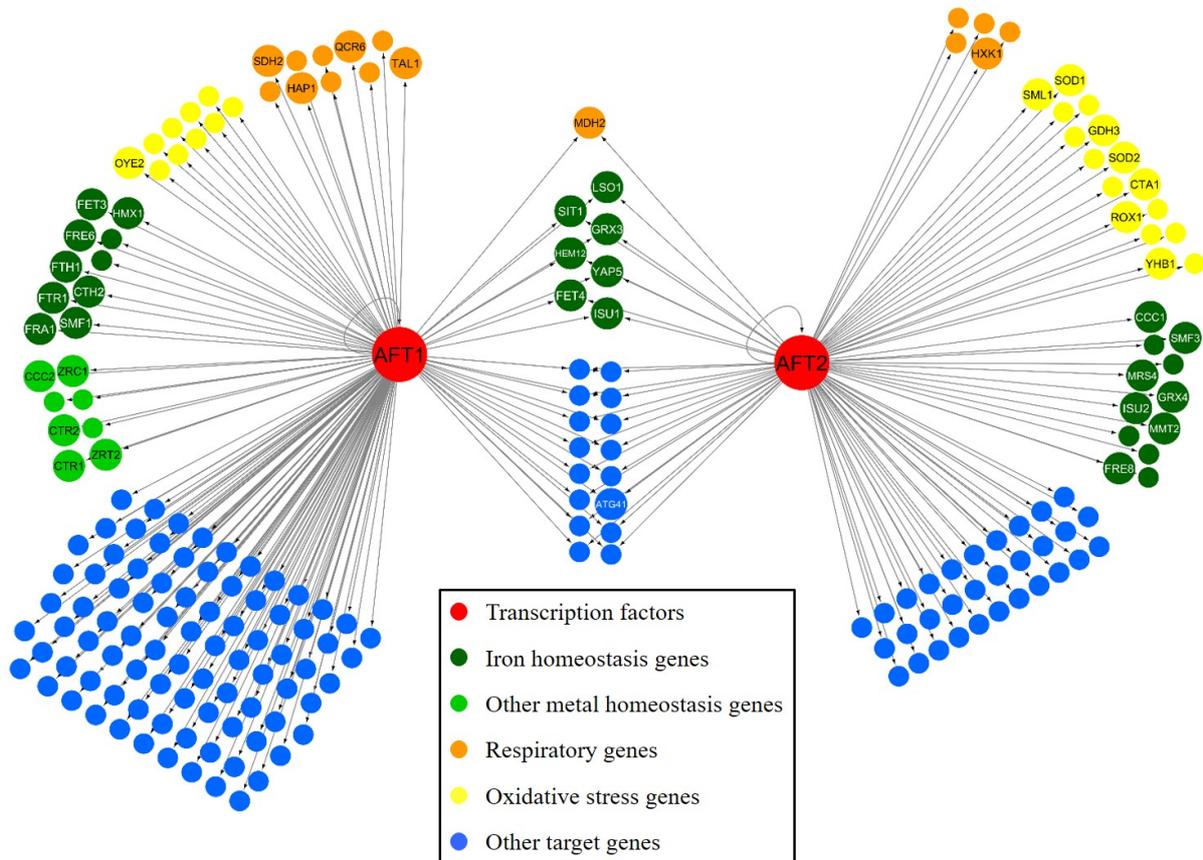


FIGURE 6.1. Visualization of Aft1 and Aft2 networks. This network was obtained with Cytoscape (Shannon et al., 2003) using Aft2 ChIP-seq results (Benchouaia et al., 2018) and Aft1 ChIP-seq results (unpublished data). As indicated, red nodes are transcription factors, dark green nodes are genes involved in iron homeostasis, light green nodes are genes related to the homeostasis of non-iron metals such as copper and zinc, orange nodes are genes involved in cellular respiration, yellow nodes are genes involved in oxidative stress response and blue nodes are all the other targets.

genes belong to the iron uptake and recycling processes pathways as a part of the system activated by Aft1 : *FTR1*, *FET3*, *SIT1*, *FTH1*, *HMX1* and *CTH2* (Figure 6.1). All these genes are bound by Aft1 in our ChIP. Interestingly, they also showed that *CTH2* is transcriptionally regulated by Aft1 and has a role of negative post-transcriptional regulator of iron consuming genes (such as *CCC1*, *MMT2*, *HEM15*, *CTA1*, *COX6*, *CYC1*, *CYT1*, *CCP1*, *ISA1*, *ACO1*, *IDH1* and *IDH2*). Gerwien et al., 2017 added another element to the list of genes under Aft1 control, namely *FRE6*, which is also among Aft1 targets in our results (Figure 6.1).

Unfortunately, further investigation of Gerwien et al., 2016 transcriptomic data didn't reveal a greater overlap between our ChIP results and their most repressed/activated genes upon Aft1 deletion. During our microarray assays, we considered that a gene is activated if the $\log_2(\text{expression ratio})$ of this gene is greater than 0.8, and repressed if it's lower than -0.8. When we applied this criteria to Gerwien et al., 2016 data, we found 681 activated genes and 563 repressed genes, among which only 34 and 36

genes, respectively, belonged to our ChIP targets. This discrepancy may have several causes. Firstly, we didn't use the same methods to achieve the iron-deficiency condition. While we only added BPS in large excess in the media, Gerwien et al., 2016 used a protocol to completely remove all iron from the growth media. This might create differences in the cell response to iron deprivation. Secondly, we chose stringent parameters ChIP peak calling, thus it is possible we missed some peaks that would correspond to genes whose expression is varying in the transcriptomic data. To answer that caveat, looser parameters were tested. A few more common peaks between ChIP and transcriptome were retrieved, but it didn't account for the huge difference between Aft1 targets in ChIP and in transcriptomic. The last possible explanation is a problem inherent to transcriptomic experiments : it is impossible to know whether the effect of a deletion of a TF on gene expression is direct or indirect. Hence the importance to combine experiments such as ChIP-seq and microarrays. For example, Gerwien et al., 2016 reported variation in expression of several genes post-transcriptionally regulated by *CTH2* (see previously). None of these genes are targeted by Aft1 in ChIP. However, *CTH2* is a target of Aft1 which regulates its expression, which explains why the deletion of Aft1 impacts the expression of *CTH2* targets without directly binding them.

Overall, despite the lack of overlap between genomic and transcriptomic data, these results tends to go in the same direction : GO analyses of top repressed/activated genes in Gerwien's data showed an enrichment in metal homeostasis genes, respiratory genes and redox genes, which are the types of targets we have in ChIP (Figure 6.1). Also, it seems to display the importance of Aft1 in iron homeostasis, as in *S. cerevisiae*. Moreover, Srivastava et al., 2015 found TGCACCC (Aft1 binding motif in the baker's yeast) to be the most enriched motif in the promoters of the genes induced during iron-deprivation. Gerwien et al., 2016 made the same statement and also found an enrichment of this motif in the genes they identified as Aft1 targets and our ChIP data confirmed this assertion : this motif is the most enriched motif in our ChIP peaks as well (61% of the peaks contain it). Additionally, it confirms the prediction of Gonçalves et al., 2014 claiming that Aft1 binding motif in *C. glabrata* was TGCACCC.

6.3.3 *C. glabrata* Aft1 has several functions shared with *S. cerevisiae* Aft1

The available data on Aft1 factor in *C. glabrata* is much more restricted than in *S. cerevisiae*. Still, we can draw a short comparison between the two species. This transcription factor is best known in *S. cerevisiae*, the species in which it was discovered. Yamaguchi-Iwai et al., 1995, showed that overexpression of Aft1 increases cell susceptibility to media containing high iron concentrations. Aft1 overexpression

wasn't studied in *C. glabrata*. Interruption of *S. cerevisiae* Aft1 causes susceptibility to iron-depleted conditions and impairs cell growth. However, they reported no growth difference for both *AFT1*-modified strains in rich growth media such as YPD. Contrary to *S. cerevisiae*, Aft1 deletion in *C. glabrata* seems to be lethal in cells cultivated in rich growth media such as YPD. To delete this transcription factor and maintain a cell line deleted for Aft1, a strong iron supplementation in the growth media is required (Gerwien et al., 2016). This implies that Aft1 is much more important to cell metabolism and survival in *C. glabrata* and that it might be essential in some conditions. This is confirmed by Srivastava et al., 2014 who weren't able to delete *AFT1* in *C. glabrata* in standard growth conditions. Similarly, in our team, Thierry Delaveau showed that *AFT1* deletion in YPD was possible only if the cells were provided with a plasmid expressing a WT version of *AFT1*.

Our ChIP-seq experiments revealed that most of the aforementioned iron homeostasis genes (such as *CCC2*, *FET3*, *FRE6*, *SIT1*, *CTH2*...) are actually bound by Aft1 during iron deprivation the same way they are in *S. cerevisiae* (*FRE6* was shown to be under the control of Aft1 in this species by Martins et al., 1998). Most of these genes belong to the pathways of high-affinity reductive iron uptake, siderophore uptake and Fe–S cluster assembly. Thus, recent data (Srivastava et al., 2014; Gerwien et al., 2016 and our ChIP experiments) tends to show that Aft1 crucial role in iron uptake during iron deprivation is conserved in *C. glabrata*. It also showed that some genes belonging in other pathways are bound by Aft1 in *S. cerevisiae* and *C. glabrata*, for example *ATG41* (autophagy), *CTR1* (copper transport), *GIP3* (glycogen metabolism), *LSM5* (mRNA processing). This is in accordance with the finding that Aft1 roles in *S. cerevisiae* are not limited to iron homeostasis : it was shown that this TF is also involved in copper and zinc metabolism (L. Li et al., 2004; Pagani et al., 2007), cell cycle and chromosome segregation (Casas et al., 1997; Measday et al., 2005), biotin metabolism, nitrogen assimilation, and purine biosynthesis (Shakoury-Elizeh et al., 2004) and pH homeostasis, cell-wall stability and DNA damage (Berthelet et al., 2010). Srivastava et al., 2014 and Srivastava et al., 2015 reported the involvement of *C. glabrata* Aft1 in response to pH variation and adherence respectively, and in a more general way, our ChIP-seq data displayed several targets involved in all the pathways previously mentioned. This might be an indication that several roles of Aft1 are conserved between *S. cerevisiae* and *C. glabrata*.

Another common point between the two species is the binding motif of Aft1. In *S. cerevisiae*, Yamaguchi-Iwai et al., 1996 showed that Aft1 favours binding to PyPuCACCC sequence, which is most of the time TGCACCC. As commented earlier, this favourite binding motif seems to be conserved in *C. glabrata*.

6.3.4 *C. glabrata* Aft1 and Aft2 roles are only partially redundant

As shown in Benchouaia et al., 2018, Srivastava et al., 2014 and Gerwien et al., 2016, *aft2* Δ cells exhibit a growth rate similar to wild type cells in standard growth conditions (YPD) as well as iron-depleted conditions. This is different from the *aft1* deleted cells, which cannot grow in standard growth media nor iron-depleted media. This might suggest that some roles are specific to each factor and raise the interesting question of the differences existing between these paralogues.

This is further confirmed by the ChIP-seq results of both factors. While Aft1 shows an enrichment in genes of transition metals homeostasis and particularly iron homeostasis, Aft2 targets are enriched in iron homeostasis and oxidative stress response (Figure 6.1). Aft2 has a majority of specific targets in several pathways. In iron homeostasis, these specific pathways are the reductive iron uptake (*FRE8*), the intracellular iron transport (*MRS4*, *SMF3*, *CCCI*, *MMT2*) and the iron-sulfur cluster biogenesis and assembly (*ISU2*, *ISD11*, *NFS1*, *YAH1*). In redox homeostasis and oxidative stress response, these specific functions are reductases (*HBN1*, *YHB1*, *RNR1*, *ERG4*...), sodium dismutases (*SOD1*, *SOD2*) and catalase (*CTA1*). Thus, it is clear that Aft2 has a more important role in oxidative stress than Aft1. This is further supported by the fact that Aft2 is regulated by Yap1 (Salin et al., 2008), a key factor in oxidative stress response and targets *ROX1*, a TF involved in response to oxygen availability. Additionally, our study disclosed some new functions for Aft2, such as a role in No Go Decay and a possible involvement in mitophagy, which haven't been listed yet as roles for Aft1.

Interestingly, Aft1 and Aft2 only share 24 common targets in ChIP. Among this common targets, a large proportion of them are known to be key genes in iron homeostasis (*FET4*, *GRX3*, *HEM12*, *ISU1*, *LSO1*, *SIT1*, *YAP5*). In these common targets, only 5 genes have a changed expression when Aft2 is deleted, while 19 genes display expression variation upon Aft1 deletion, which can let us think that Aft1 is the main regulator of these genes despite the fact they also are Aft2 targets.

ChIP-seq experiments revealed two other interesting features. Firstly, it is likely both factors are submitted to an autoregulation phenomenon, given the fact they are targeting their own promoters. Secondly, Aft1 and Aft2 shares a core binding motif, namely CACCC. Indeed, Aft1 tends to bind on TGCACCC sequences and Aft2 tends to focus on CACCC sequences. The resemblance of the binding motifs might explain why some targets are shared between the two factors.

6.3.5 Relationship between Aft factors in *S. cerevisiae* share some features with *C. glabrata*

Blaiseau et al., 2001 and Rutherford et al., 2001 showed that sole deletion of Aft2 doesn't have iron-dependent phenotype in *S. cerevisiae*, which resembles *C. glabrata*. Accordingly, our Aft2-myc ChIP data showed the conservation of several targets compared to *S. cerevisiae* : as displayed in the previous article, our study of Aft2 targets demonstrated an enrichment in genes involved in iron homeostasis (reductive iron uptake pathway, siderophore iron uptake pathway, intracellular iron transport and iron-sulfur cluster biogenesis), oxidative stress response (reductase enzymes, sodium dismutase enzymes and catalase enzymes), which resembles *S. cerevisiae*.

Rutherford et al., 2001 reported that double deletion of Aft1 and Aft2 renders cells more sensitive to low-iron conditions than the single *aft1*. So far, the double deletion of Aft1 and Aft2 hasn't been carried out in *C. glabrata*, so we cannot compare yet the impact in this species. This implies that the Aft factors have a partially redundant role in *S. cerevisiae* and thus, some common target genes. This was confirmed by Rutherford et al., 2003 and Courel et al., 2005, who showed that reductases (*FRE1-2*), permeases (*FTR1*, *FET3*), iron transporters (*FIT1-3*, *CCC2*) and genes promoting mRNA degradation (*CTH2*), among other genes, are common targets of the Aft factors. These common genes between the Afts are mostly not conserved in *C. glabrata*, except for a few genes such as *LSO1* or *ISU1*, even if we still find some genes of the same families conserved in *C. glabrata* (for example, *FET4* instead of *FET3*).

The fact that some targets are shared by the two factors can be represented by the similarity of the Aft binding motifs : TGCACCC for Aft1 (Yamaguchi-Iwai et al., 1996) and ACACCC for Aft2 (Courel et al., 2005; Silva et al., 2009). In all the studies of the binding motifs, the core motif seems to be CACCC, which might allow the binding of one factor on the targets of the other. However, it does not prevent some genes to be specifically activated by one factor, for example Aft1 is specifically activating genes involved in cell surface iron uptake systems (Rutherford et al., 2003; Courel et al., 2005) (*ARN1*, *SIT1*...) while Aft2 is specifically regulating vacuolar iron storage (Rutherford et al., 2001) and mitochondrial iron transport, subcompartmentation and use (*SMF3*, *MRS4*...) (Courel et al., 2005). Thus, the binding motifs of the Aft factors are conserved between *S. cerevisiae* and *C. glabrata*, and so is a part of the specific targets of Aft1 or Aft2.

However, interestingly, overexpression of Aft2 in *aft1Δ* cells partially compensate the growth deficiency in iron-dependent conditions and oxidative stress conditions (Blaiseau et al., 2001; Castells-Roca et al., 2011). This was confirmed in Courel et al., 2005, who showed that the amount of one Aft factor

is increased in the absence of its paralog under iron depletion. In other words, in iron-limiting condition, when an Aft factor is absent, the other Aft is overexpressed to compensate for the lack of gene activation during iron-deficiency response. This might suggest that there is a cross-regulation between Aft1 and Aft2, at least at the proteic level. This situation is trickier in *C. glabrata* : ChIP experiments revealed that each Aft is binding its own promoter, which lets think of an autoregulation of each factor. This is confirmed by the fact that *AFT2* deletion doesn't impact Aft1 expression in iron-depleted media. However, Aft2 is activated in aft1-deleted cells upon encounter of iron-limiting media, which doesn't fit with the theory of autoregulation, and required more investigations.

6.3.6 Relationship between Aft factors in more distant species

In the other post-WGD species, we can also find two Aft factors, and they both kept the same favourite binding motifs : Aft1 has a strong affinity to TGCACCC while Aft2 favours ACACCC (Courel et al., 2005; Silva et al., 2009; Gonçalves et al., 2014; Srivastava et al., 2015; Gerwien et al., 2016). Most likely, we can suppose that the Aft factors kept their role in iron homeostasis in these species. In pre-WGD species, such as *Kluyveromyces lactis*, there is only one Aft factor, KIAft. It was identified by (Silva et al., 2009) thanks to its high resemblance in proteic domains with both *S. cerevisiae* Aft1 and Aft2. The deletion of KIAft had the same impact than the deletion of Aft1 and Aft2 in *S. cerevisiae* in iron depleted conditions and the insertion of Aft1 into Kiaft Δ only partially restored the phenotype. They also noted that KIAft controlled the expression of several orthologues of cell surface iron transport genes in *S. cerevisiae* but not intracellular iron transporter genes. To put it another way : KIAft controls orthologues of Aft1 targets but not of Aft2 targets. However, they reported that KIAft was binding the ACACCC sequence (Aft2 binding motif) in its target genes, which means that KIAft has the same role than Aft1 but binds to the Aft2 motif. Gonçalves et al., 2014 performed a more precise study of Aft binding motif in species from *S. cerevisiae* to *K. lactis*. They found orthologues of *S. cerevisiae* Aft1 targets in all the species (although *K. lactis* lacks any Cth2 ortholog) which might let us think that Aft conserved its role in these species. Moreover, they found that TGCACCC is the most enriched motif in the orthologues in all species but *K. lactis*. It suggests that TGCACCC is the ancestral Aft-binding site and that it diverged toward ACACCC in *K. lactis* and *S. cerevisiae* Aft2.

In species even more distant of *S. cerevisiae* than *K. lactis*, such as *Candida albicans*, the iron deprivation response is mainly carried out through three transcription factors : Sef1 (Homann et al., 2009; C. Chen et al., 2012), Hap43 (Baek et al., 2008; C. Chen et al., 2011) and Sfu1 (Lan et al., 2004; Singh et al.,

2011). Liang et al., 2010 found one sequence homologous to *S. cerevisiae* Aft2, termed CaAft2. They reported that CaAft2 can save *S. cerevisiae aft1Δ* growth defect under iron depleted conditions, which means that CaAft2 has the same role than Aft1. However, they also showed that deletion of CaAft2 had no impact on cell growth in iron depleted conditions. Most likely, it was caused by compensatory mechanisms from the other iron acquisition pathways in *C. albicans*. They proved that CaAft2 has a role in ferric reductase activity which also resembles Aft1 activity in *S. cerevisiae*. They also found that CaAft2 had a role on cell morphology and invasive growth. Finally, they reported the finding of several TGCACCC motifs in ferric reductase genes such as *FRE2* or *FRP1*. Thus, it seems that CaAft2 shares common features with *S. cerevisiae* Aft1. Interestingly, Xu et al., 2012 showed that CaAft2 was a transcriptional repressor of *MRS4* through the core CACCC motif. It means that *MRS4* is still regulated by Aft2 in *C. albicans*. Nonetheless, it is the first and only report so far of the negative action of Aft factors, which always play the role of genes activators. Xu et al., 2013 deciphered more clearly this dual role of CaAft2: it both activates genes such as *FRP1*, *FET3*, *FTR1*... (Aft1 targets in *S. cerevisiae*) and represses genes such as *MRS4*, *SMF3*... (Aft2 targets in *S. cerevisiae*). They also showed that CaAft2 has a role in oxidative stress response. Overall, it seems that CaAft2 shares a lot of features with both *S. cerevisiae* Aft1 and Aft2 : common target genes, common binding motif, role in iron homeostasis oxidative stress, pH and cell wall stability, same phenotypic impact upon deletion. However, it also strongly differs from *S. cerevisiae* in its transcriptional regulation abilities : whereas Aft factors in *S. cerevisiae* and all the other species are transcriptional activators only, CaAft2 is both a transcriptional activator and repressor.

6.3.7 GRX3 and GRX4 are involved in iron-deprivation response under different regulations

GRX3 and *GRX4* are both bound by Aft2 as showed in the ChIP-seq data of the article. The deletion of *AFT2* only impacts *GRX3* expression, while *GRX4* expression remains unchanged (supplementary data of the article). In our ChIP-seq data (unpublished), *GRX3* is also bound by Aft1, but not *GRX4*. Also, Gerwien et al., 2016 reported no change in *GRX3* nor *GRX4* expression when *AFT1* was deleted. When we take into account the results of the previous part (namely *GRX4* is regulated by the Hap5-Yap5 system, whereas *GRX3* isn't), it could show an interplay between the Aft regulation and the Hap5-Yap5 regulation (Figure 6.2).

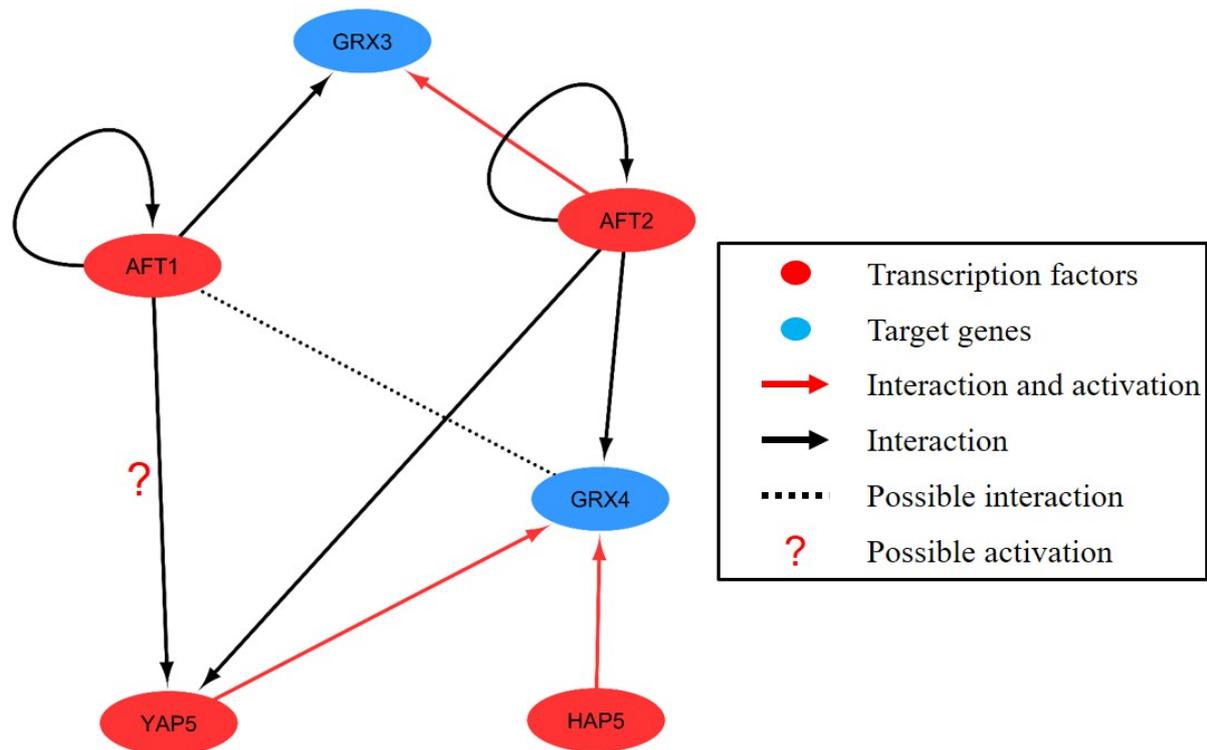


FIGURE 6.2. Visualization of the links between *GRX* genes and several transcription factors. This network was obtained with Cytoscape (Shannon et al., 2003) using data mentioned previously. As indicated, red nodes are transcription factors, blue nodes are target genes *GRX3* and *GRX4*, a red arrow indicates an activating interaction, a black arrow indicates an unknown direction of regulation and a dotted arrow indicates a possible interaction.

We put in parallel these results and the presence of Aft1, Aft2, Hap5-Yap5 binding sites in *GRX3* and *GRX4* across 28 Saccharomycetaceae species (Figure 6.3). The first thing to notice is the repartition of Aft binding motifs and CCAAT-YRE binding motifs : in pre-Whole Genome Duplication species, we find a majority of CCAAT-YRE sites in *GRX3/4*. However, in species who underwent the WGD, most of the CCAAT-YRE are found in *GRX4* genes, while the Aft sites are found in *GRX3*. This seems to be almost exclusive : you can find one type of sites or the other, but rarely both types in the same gene. A major exception to this statement is found in *Naumovozyma castelli*, where one gene gathers all the motifs and the other is totally empty of them. Unfortunately, BLAST results didn't give significant clues about the identity of this gene, so we still don't know whether this gene is *GRX3* or *GRX4*. This repartition of presence/absence of binding sites appears quite logical with the ChIP results in mind : there are Aft1 and Aft2 sites in *GRX3*, and we showed this gene was found in the ChIP targets of both factors.

Conversely, *GRX4* contains CCAAT-YRE motifs and is bound by Hap5 and Yap5. However, it also holds an Aft1 site, which seems to be unused given we didn't find *GRX4* as a target of Aft1. Finally, there is an Aft2 site in *GRX4*. This site is used because we showed with ChIP that this gene is targeted by Aft2. However, the deletion of Aft2 has no effect on *GRX4* expression, which could mean this gene is

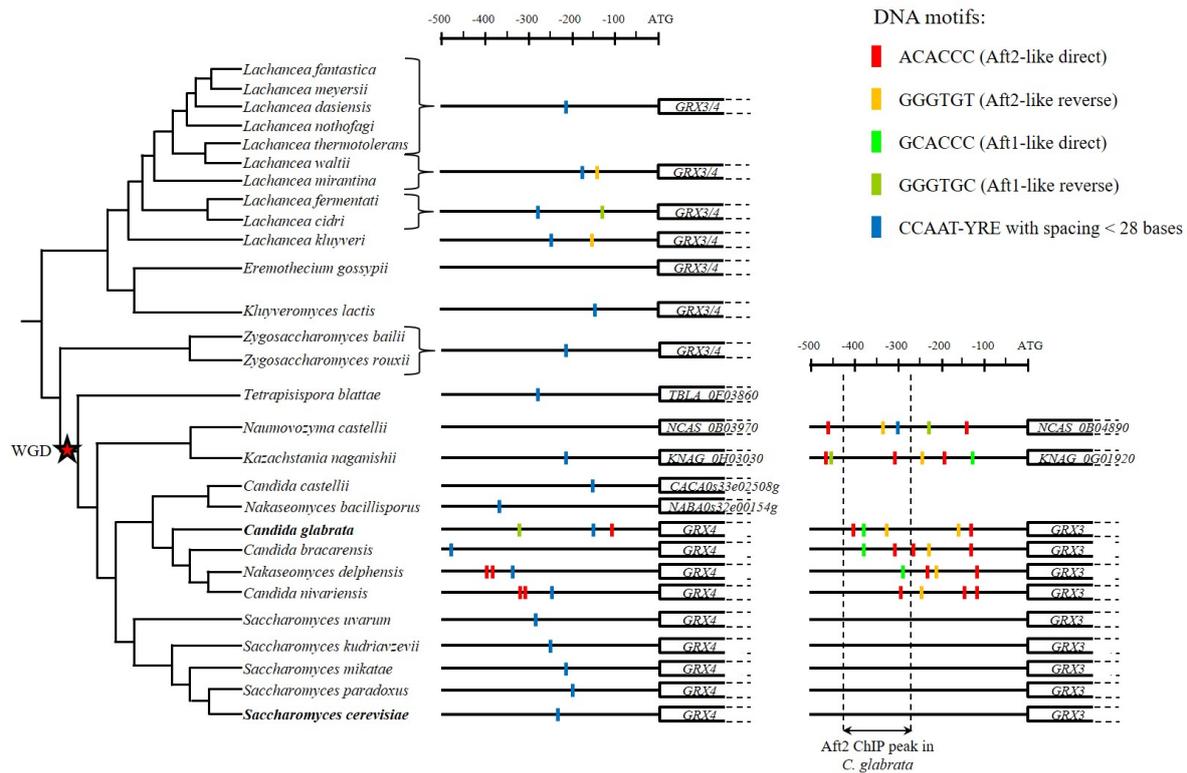


FIGURE 6.3. Analyses of the promoters of the *GRX3/4* orthogroup in 28 *Saccharomycetaceae* species. The tree is a schematic representation of the phylogeny of the species inspired from Vakirlis et al., 2016 for the *Lachancea* species and from X.-X. Shen et al., 2016 for all the other species. The Whole Genome Duplication (WGD) is indicated by a red star. The positions of the Aft1/Aft2/Hap5/Yap5 binding sites in the promoters of the genes are indicated by colored boxes. Color code is on the upper right part. As previously, only the CCAAT and YRE motifs spaced by a maximum of 28 bases are displayed. The boundaries of the Aft2 ChIP peak in the *C. glabrata GRX3* gene are indicated by dashed vertical lines. This figure was adapted from Fig S14 of the article.

subjected to several regulations at the same. An obvious answer could be the Hap5-Yap5 regulation. In this case, the Hap5-Yap5 regulation would be “epistatic” to the Aft2 regulation, even though testing such an hypothesis would require more experiments. Another explanation of the absence of effect of Aft2 deletion on *GRX4* expression could be the existence of spurious binding (Spivakov, 2014).

These differences in binding sites and regulation between *GRX3* and *GRX4* might be the start of differentiating the functions of the two genes. Until now, the studies suggested that these two proteins are very much alike, have the same cellular location and have the same function (Pujol-Carrion et al., 2006; Mühlenhoff et al., 2010; Ueta et al., 2012; Poor et al., 2014; Martínez-Pastor et al., 2017). However, most of the time the authors are studying only one of the proteins and just assume that the other protein will behave in the same way because of their similar structure. Even if they do have a lot in common, this might not be perfectly true. For example, the previous results indicate that in *C. glabrata*, Grx3 belongs to the Aft regulation of iron homeostasis, whereas Grx4 is as the crossroads of Aft and Hap5-Yap5

regulation. In the same vein, Abdalla et al., 2018 recently showed that Grx3 and Grx4 have different patterns of expression during the cell cycle. Grx3 is mainly expressed during the M stage, while Grx4 expression is greater, especially in the G1-S stages. This kind of results let us think that Grx3 and Grx4 might not be as similar as we think presently and that we just haven't found yet their differences.

Additionally, we also found that *YAP5* is a target of Aft1. Yap5 is strongly induced during iron-deficiency (Gerwien et al., 2016; Merhej et al., 2016), however the log₂(expression) of Yap5 in iron deficiency in the microarrays of Gerwien et al., 2016 drops from 2.9 to 1.3 (supplementary data of Gerwien et al., 2016). Basically, Yap5 induction is partially lost when Aft1 is deleted, which could mean that Aft1 induces Yap5 in this condition. This would clearly exhibit a link between the Aft regulation and the Hap5-Yap5 regulation (Figure 6.2). However, the deletion of Aft2 doesn't impact the expression of Yap5 (previous manuscript).

6.3.8 Conclusion

These supplementary data clearly exhibited an intricate network (or even networks) in iron homeostasis. Besides the key role of *GRX* genes, the impact of the Hap5-Yap5 system on *GRX4* and the impact of the Aft1-Aft2 on *GRX3* and possibly *GRX4*, it becomes more and more obvious that these two transcriptional regulation systems are linked. Interestingly, while we highlighted the likely conservation of the Hap5-Yap5 system in the previous part, we discussed and illustrated the strong conservation of the Aft system in this part. Indeed, the Aft system appears to be quite conserved between *S. cerevisiae* and *C. glabrata*. It is likely conserved in other post-WGD species, and displayed some common features with pre-WGD species such as *K. lactis*, and even further species such as *C. albicans*. Finally, the existence of a double transcriptional role in *C. albicans* raise another exciting question : was the repressing role of Aft in *C. albicans* acquired or was it lost during evolution of the other species ?

Conclusion and perspectives

This manuscript allowed me to present the work I have been doing during my PhD for the last three years. It can be summarised in three main parts.

Overall, we performed high-throughput ChIP-seq experiments on 6 transcription factors (Aft1, Aft2, Hap4, Hap5, Rox1 and Skn7) in stress conditions designed to activate the binding of said TFs : iron starvation for Aft1 and Aft2, YPD and non-fermentable media for Hap4, YPD for Hap5 and Rox1 and osmotic and oxidative stresses for Skn7. These are the first data of this type on these TFs in *Candida glabrata*. Moreover, this largely increases the available ChIP-seq data in this yeast : a search on the NCBI-GEO database reveals that the genome occupancy of only 3 TFs was studied by ChIP-seq in *C. glabrata* (without taking into account the TFs previously studied by the lab). Hence, this work triples the number of studied TF. Besides, among the 5 datasets available on genome occupancy, two of them originate from my PhD (and another one originate from the team). This displays the importance and the strength of the Candihub project in providing high-throughput genome occupancy data on *C. glabrata*. Additionally, we generated expression data on the impact of the deletion of *AFT2* and *HAP5* in several stress conditions. All these data are likely to help the scientific community working on *Candida glabrata*. More data will come, as the team will perform ChIP-seq on Mac1 (a TF involved in copper homeostasis), Zap1 (a TF involved in zinc homeostasis) and Pdr1 (a TF involved in *C. glabrata* drug resistance). This will further reinforce the usefulness of the Candihub project and my PhD.

When we combined these data with previous ChIP-seq data of the team on the Yap family of transcription factors, we were able to reconstruct a rich regulatory network of interactions describing the stress responses of *Candida glabrata*. This network comprises 12 TFs and more than a 1000 genes and almost 2000 interactions. It is the biggest network reconstructed in *C. glabrata* using only experimental data. Thanks to this network, we were able to identify 12 genes at the core of the network. In all likelihood, these genes are essential for the yeast to adapt or to survive a range of various stresses. Interestingly, three of these genes (*HEM3*, *CCC1*, *SIT1*) are involved in iron homeostasis. This result might be biased because we studied several TFs involved in iron homeostasis. However, these genes are equally bound by TF known to be involved in iron homeostasis and TF unrelated to iron homeostasis, though some TFs are close to iron metabolism, for example Yap1 in oxidative stress. In any case, these genes are targeted by many TFs and thus are at the crossroads of several key pathways. This shows how important they are for *C. glabrata*. Accordingly, these genes required to be investigated more thoroughly.

Still, many things remain to be unraveled. We produced an extensive amount of data that can be investigated even deeper. For example, a possible interesting study could reside in detailing Skn7 functions. Skn7 is known in *S. cerevisiae* to be involved in oxidative and osmotic stress responses. Given the targets obtained in our ChIP-seq, these roles might be conserved in *C. glabrata*. In particular, we noticed that there are a lot of genes encoding cell wall proteins and cell wall surface proteins such as GPI-anchor proteins among the targets. One of them is Rlm1, which is involved in cell wall building and maintenance in *S. cerevisiae*. This could show a link between Skn7 and the cell wall. This is further strengthened by the fact that Rlm1 has a paralog, Smp1, which is induced in osmotic stress. Despite not being directly bound by Skn7, Smp1 is under the control of transcription factors Hog1 and Rim101, which are targets of Skn7 in *S. cerevisiae*. To clarify this situation, I performed ChIP-seq experiments on Rlm1 and Smp1 in conditions of cell wall stress. Unfortunately, these experiments failed, maybe because of the conditions that were used. This needs further investigations. Additionally, I built strains deleted for *SKN7* and *RLM1*, *SKN7* and *SMP1* and *RLM1* and *SMP1*. Unluckily, I didn't have time to experiment on them with microarrays and this also needs further focus. Thus, Skn7 seems to be an compelling lead for future prospects.

On one hand, on top of the ChIP-seq results, we also focused on the role of the CCAAT Binding Complex in *C. glabrata*. This complex had never been studied in this yeast before. We demonstrated that the CBC has a double role in cellular respiration and iron homeostasis, depending on its interacting regulatory proteins. The CBC and Hap4 are responsible for the activation of the respiratory genes when the respiratory pathway is needed and the CBC-Yap5 complex is activating iron consuming genes in case of iron excess. This latter regulation is highly dependant on the presence of both Hap5 and Yap5 binding motifs, namely CCAAT and TTACTAA, as the loss of one motif impairs the activation of iron excess genes.

This double role of *C. glabrata* CBC is new and particularly interesting, because it is in-between the CBC role in *S. cerevisiae* (respiration) and the CBC role in fungi such as *C. albicans* (iron homeostasis). This model might even bridge the gap on the CBC role existing between *S. cerevisiae* and further yeast species. While the CBC was thought to be mainly responsible for the respiratory pathway in *S. cerevisiae*, it might not stand true : all the *C. glabrata* targets of the CBC and Yap5 have orthologues in *S. cerevisiae*. Some of these targets were already demonstrated to be involved in iron excess reponse in *S. cerevisiae* and regulated by Yap5, exactly like in *C. glabrata*. Additionally, the promoters of these target genes present both Hap5 and Yap5 binding motifs with a conserved spacing between them, compared to *C.*

glabrata. Even more striking, some of these promoters are actually bound by Hap5 in *S. cerevisiae* (demonstrated by ChIP-chip) during growth in rich media. The relationship between Hap5 and these specific targets was never studied in iron excess, but this could be the sign that the CBC and Yap5 also play a role during iron excess response in *S. cerevisiae*. Further species such as *Lachancea kluyveri* and *Kluyveromyces lactis* also display orthologues of *C. glabrata* iron excess genes, conserved regulation between Yap5 and a part of these targets and conserved binding motifs and spacing between those motifs. Even if these proofs are weaker than in *S. cerevisiae*, it is possible that the CBC-Yap5 role in iron excess is conserved in *L. kluyveri* and *K. lactis*. This means that the involvement of the CBC and Yap5 during iron excess response is more frequent than what was thought previously. It also means that several yeast species between *S. cerevisiae* and *C. albicans* share the same mechanisms, including pathogenic and non-pathogenic species. As a consequence, despite being crucial in iron excess response, the CBC-Yap5 complex is unlikely to be a specific feature of *C. glabrata* pathogenicity. Nevertheless, this needs to be further confirmed, for example by studying the link between the CBC and virulence in *C. glabrata*.

On the other hand, we investigated the iron-starvation response of *C. glabrata*, and especially the role of Aft factors in it. Aft1 was previously studied using transcriptomic approaches and we completed this set of data with the first ChIP-seq ever performed on *C. glabrata* Aft1. Our ChIP-seq experiments confirmed the findings of the previous teams about the targets, the GO enrichment among the targets and the binding motif. More important, it definitely confirms the crucial role of Aft1 during iron-starvation response. This group of studies also highlighted the common points between *C. glabrata* and its orthologue in *S. cerevisiae* : targets, role and binding motif are very similar in both species. These studies also allowed to show the closeness between *C. glabrata* Aft1 and Aft in pre-Whole Genome Duplication species.

We also studied the role of Aft1 paralogue, Aft2, during iron-starvation response using transcriptomic and genomic approaches, which is entirely new because this TF was never studied before in *C. glabrata*. We were able to identify Aft2 regulatory network during iron-starvation response, as well as Aft2 binding motif. This displayed a few targets shared with *C. glabrata* Aft1 (as well as their roles), but it especially emphasized that Aft factors have dissimilar roles in *C. glabrata*. *C. glabrata* Aft1 and Aft2 have more differences than *S. cerevisiae* Aft1 and Aft2, despite the fact that Aft2 has the same binding motif and common targets in both species. This was particularly clear when we were able to identify four genes specifically regulated by Aft2 in *C. glabrata* under iron starvation, compared to *C. glabrata* Aft1 and *S. cerevisiae* Aft2 networks. Among these four genes, two are required for optimal growth in iron

starvation conditions. The regulation of these two genes likely arose from the WGD, and is only found in *C. glabrata* and its sister *Candida* species. Interestingly, this clade is enriched in pathogenic species, which might show a link between this new-found regulation and pathogenicity. This could make sense given that iron homeostasis is a key virulence factor. However, we need to check for the conservation of this Aft2-dependant regulation under iron starvation in other species between *S. cerevisiae* and *C. glabrata* to draw a conclusion. In any case, it shows that Aft factors have evolved new functions and transcriptional regulations in *C. glabrata* compared to *S. cerevisiae*.

As a conclusion, this work permitted to gain a huge knowledge into *C. glabrata* iron homeostasis by unraveling mechanisms of the unknown iron-excess response and completing previous data on the iron-starvation response. Additionally, and maybe more importantly, it gave new information on the evolution of the transcriptional regulatory networks of the Aft factors and the CBC-Yap5 complex and brought new data on the relationship between iron homeostasis and pathogenicity. However, a lot remains to be understood.

These data also show that networks reconstructed with a combination of transcriptomics and genomics experiments are reliable. However, genomics experiment like ChIP-seq are still very expensive and long to perform. There may be a way to bypass these problems with the use of softwares such as REDUCE (Foat et al., 2006). The principle of REDUCE is the following : it takes as input transcriptomics data and promoter sequences and then uses a mathematical model to associate expression variations to DNA motif of 5 to 12 bases. It is easy to locate these motifs in the genome and associate them with promoters or genes. They can also be entered in databases (such as Yeastract for yeast) to check whether these motifs are already linked to a known transcription factor. The combination of several types of informations (gene expression, binding motif, "target" genes, link to a known TF...) could lead to interesting results while being much more affordable than genomics studies.

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Appendices

**Network of Stress Response Transcription
Factors in *Candida glabrata***



A Network of Paralogous Stress Response Transcription Factors in the Human Pathogen *Candida glabrata*

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The yeast *Candida glabrata* has become the second cause of systemic candidemia in humans. However, relatively few genome-wide studies have been conducted in this organism and our knowledge of its transcriptional regulatory network is quite limited. In the present work, we combined genome-wide chromatin immunoprecipitation (ChIP-seq), transcriptome analyses, and DNA binding motif predictions to describe the regulatory interactions of the seven Yap (Yeast AP1) transcription factors of *C. glabrata*. We described a transcriptional network containing 255 regulatory interactions and 309 potential target genes. We predicted with high confidence the preferred DNA binding sites for 5 of the 7 CgYaps and showed a strong conservation of the Yap DNA binding properties between *S. cerevisiae* and *C. glabrata*. We provided reliable functional annotation for 3 of the 7 Yaps and identified for Yap1 and Yap5 a core regulon which is conserved in *S. cerevisiae*, *C. glabrata*, and *C. albicans*. We uncovered new roles for CgYap7 in the regulation of iron-sulfur cluster biogenesis, for CgYap1 in the regulation of heme biosynthesis and for CgYap5 in the repression of *GRX4* in response to iron starvation. These transcription factors define an interconnected transcriptional network at the cross-roads between redox homeostasis, oxygen consumption, and iron metabolism.

Keywords: yeast, Yap, ChIP-seq, transcriptome, regulatory networks, evolution

INTRODUCTION

Candida glabrata is a unicellular yeast from the Hemiascomycetes phylogenetic group and a commensal host of the human mucosal microbiota. However, in patients with severe immunodeficiency, it can cause invasive systemic infections, with high mortality rates (about 40–60%). While *Candida albicans* remains the main cause of systemic candidiasis (50–70%), *C. glabrata* ranks second (20–25%), and its prevalence has increased in the last decades (Perlroth et al., 2007). An important prerequisite to the acquisition of virulence traits in *C. glabrata* is its

ability to adapt and be resistant to environmental variations, which allows the pathogen to colonize many different niches and organs in the human body, with very different features in terms of pH, temperature, redox potential, iron, zinc, or oxygen availability, etc. (Domergue et al., 2005; Nevitt and Thiele, 2011). Moreover, *C. glabrata* is able to survive and grow in the harsh environment of the phagolysosomes of macrophages (Seider et al., 2011, 2014).

The mechanisms that allow these adaptations rely partially on transcriptional regulatory networks. Systematic combination of transcriptome analyses of loss of function mutants for different transcription factors and of genome-wide chromatin immunoprecipitation has led to the comprehensive description of genome-wide transcriptional regulatory networks in the model yeast *Saccharomyces cerevisiae* (Babu et al., 2004; Harbison et al., 2004; Teixeira et al., 2014). These global approaches have also been extensively used in *C. albicans*. In this species, more than 75 specific transcription factors (over a total of about 150) have been analyzed by genome-wide approaches and comprehensive network-based models are available for ribosome biogenesis, iron homeostasis, or biofilm formation (Lavoie et al., 2010; Chen et al., 2011; Nobile et al., 2012; Fox et al., 2015). In contrast, the global transcriptional regulation in *C. glabrata* has been poorly studied. As of 2016, only 10 transcription factors have been analyzed on a genome-wide scale in this species (Vermitsky et al., 2006; Lelandais et al., 2008; Roetzer et al., 2008, 2011; Kuo et al., 2010a; Caudle et al., 2011; Ferrari et al., 2011; Miyazaki et al., 2013; Noble et al., 2013; Paul et al., 2014; Merhej et al., 2015; Wu et al., 2015). While *C. glabrata* and *C. albicans* share a common ecological niche and are from the same genus, they are very distant species according to their genomic sequence, and their ability to successfully infect humans involves quite different strategies (Brunke and Hube, 2013). For instance, in contrast to *C. albicans*, the ancestor of *C. glabrata* and *S. cerevisiae* experienced a whole-genome duplication event. In addition, *C. albicans* is usually diploid, switching frequently from yeast to hyphal growth under stress conditions, while *C. glabrata* is strictly haploid and grows mostly in the yeast form. Therefore, the *C. glabrata* transcriptional networks cannot be simply inferred from the knowledge acquired in *C. albicans* (Gabaldon and Carrete, 2015).

In the present work, we conducted a network-based analysis of the seven transcription factors belonging to the Yap (Yeast AP1) family in *C. glabrata*. The Yap proteins belong to the pap subfamily of bZIP transcription factors and are homologous to the CREB, ATF2, and Fos/Jun transcription factors of vertebrates (Fujii et al., 2000; Reinke et al., 2013). The model yeast *S. cerevisiae* has 8 Yap members (named ScYap thereafter; Supplementary File S1), most of which are involved in adaptation to environmental changes (Rodrigues-Pousada et al., 2010). ScYap1 is the major regulator of oxidative stress responses caused by reactive oxygen species (ROS), metals and drugs (reviewed in Rodrigues-Pousada et al., 2010). ScYap2 (also named Cad1), the ohnolog of Yap1, is involved in cadmium resistance (Hirata et al., 1994; Azevedo et al., 2007; Mazzola et al., 2015). The role of ScYap3 is unknown but it has been shown to contribute to the resistance to benzenic compounds and to 6-Nonadecyonic acid

(North et al., 2011; Xu et al., 2012; Adeboye et al., 2014). The ScYap4 (also named Cin5) and ScYap6 ohnologs are involved in salt stress response (Mendizabal et al., 1998; Nevitt et al., 2004; Ni et al., 2009). ScYap5 is responsible for the activation of the high iron stress response (Li et al., 2008; Pimentel et al., 2012). ScYap7, the ohnolog of ScYap5, was recently found to be a repressor of the nitric oxide oxidase encoding gene *YHB1* (Merhej et al., 2015) and ScYap8 is involved in the response to arsenic (Bobrowicz et al., 1997; Amaral et al., 2013; Kumar et al., 2015).

C. glabrata has 7 Yap members, which slightly differ from the *S. cerevisiae* family (Supplementary File S1). Orthologues of Yap1 (*CAGL0H04631g*), Yap2 (*CAGL0F03069g*, named CgYap1 thereafter), Yap5 (*CAGL0K08756g*, named CgYap5 thereafter), and Yap7 (*CAGL0F01265g*, named CgYap7 thereafter) are present, but Yap8 is absent. Two versions of Yap3 (*CAGL0K02585g* and *CAGL0M10087g*, named CgYap3a and CgYap3b thereafter) and only one ortholog for the Yap4 and Yap6 pair (*CAGL0M08800g*, named CgYap4/6 thereafter) are found. Only three of these Yaps have been studied previously in *C. glabrata*. CgYap1 has been shown to be involved in oxidative stress response, with a set of targets which is significantly conserved compared to ScYap1, but with different DNA binding preferences (Chen et al., 2007; Cuellar-Cruz et al., 2008; Lelandais et al., 2008; Kuo et al., 2010a; Goudot et al., 2011; Roetzer et al., 2011). CgYap7 has been shown to have a conserved role in nitric oxide oxidase repression (Merhej et al., 2015). Finally, as in *S. cerevisiae*, CgYap5 is involved in the activation of the *CCC1* and *GRX4* genes under high iron conditions (Merhej et al., 2015). In the present work, we conducted chromatin immunoprecipitation experiments followed by high-throughput sequencing (ChIP-seq) and transcriptome analyses to determine the targets for the seven Yap transcription factors of *C. glabrata*. The CgYap network included 309 genes and 255 regulatory interactions. From these results, we could predict with high confidence the preferred DNA binding sites for 5 of the 7 CgYaps and show a strong conservation of the Yap DNA binding properties between *S. cerevisiae* and *C. glabrata*. We provided functional annotation for 3 of the 7 CgYaps and identified for Yap1 and Yap5 a core regulon which is conserved in *S. cerevisiae*, *C. glabrata*, and *C. albicans*. Our data pointed out new roles for CgYap7 in the regulation of iron-sulfur cluster biogenesis, for CgYap1 in the regulation of heme biosynthesis and for CgYap5 in the repression of *GRX4* in response to iron starvation.

MATERIALS AND METHODS

Strains

The list of the strains used in this study is available in Supplementary File S2. All the strains were derived from the Δ HTU parental strain (Kitada et al., 1995). The genomic myc-tagging and deletion of the different *CgYAP* was performed as described previously (Merhej et al., 2014, 2015). Briefly, deletion or myc-tagging cassettes were PCR amplified from the M. Longtine's plasmids (Longtine et al., 1998) with oligonucleotides containing in 5' homology sequences flanking the desired genomic insertion points. At least 10 micrograms of purified PCR product was used to transform Δ HTU cells using a standard yeast

transformation protocol (Merhej et al., 2015). Genotyping of the clones growing on selective media was done by PCR. The PCR-verified clones for the knock-out were then verified by southern blot (Merhej et al., 2015). The correct myc-tagging of the *CgYAP* was verified by sequencing of the gene and western blot (Merhej et al., 2015). All the oligonucleotides used for cassette preparation and genotyping are listed in Supplementary File S2.

Yeast Cultures and Growth Conditions

All cultures were conducted in a rotative shaker at 30°C in YPD (Glucose 2%, yeast extract 1%, Bactopeptone 1%). Stress conditions used were: 1 mM sodium selenite, 1 M NaCl, 2 mM cadmium chloride, 5 mM iron sulfate, or 0.5 mM bathophenanthroline disulfonate (BPS). These doses were chosen, based on preliminary microarray and growth assay experiments, to induce a transcriptional response in the wild type without causing significant differences of growth rates between the wild type and the *CgYAP* knock-out mutants (data not shown).

Chromatin Immunoprecipitation and High-Throughput Sequencing

For ChIP, myc-tagged *CgYAP* strains were grown in YPD until exponential phase (OD = 0.8) and then stressing agents were added for 30 min. Cross-linking of the cells and ChIP were performed as described previously (Lelandais et al., 2016). The parental Δ HTU (untagged strain) was grown and processed the same way to provide the mock-IP samples. Sequencing of the IPs, Input DNAs and mock IPs samples and primary data analyses (quality controls and mapping of the reads) were performed as described previously (Lelandais et al., 2016). All experiments were performed twice and the reads of the replicate averaged before the peak calling step, except for *CgYap5* for which one of the two replicates had poor read coverage and was not used for further analyses. Peak calling was performed with the bpeaks software (Merhej et al., 2014), using both the Input DNA and the mock IP as references. For peak calling using the Input DNA as reference, the bpeaks parameters were T1 = 2, T2 = 2, T3 = 1.5, T4 = 0.7. For peak calling using the Mock IP as reference, the bpeaks parameters were T1 = 2, T2 = 2, T3 = 1.5, and T4 = 0. Only the peaks which were found by the two analyses were kept for further processing. These peaks were then manually checked on a genome browser (Thorvaldsdottir et al., 2013) to discard artifactual peaks (e.g., peaks centered on a tRNA locus or perfectly overlapping a highly expressed ORF) which would have escaped the bpeaks filter (Supplementary Files S3, S4). The ChIP seq data can be downloaded from the GEO database (accession number: GSE77904).

Transcriptome Analyses

Knock-out and wild type strains were grown in 50 mL of YPD until exponential phase (OD = 0.8) and then stressing agents were added. After 30 min, 20 mL of each cell cultures were flash-frozen in two volumes of cold ethanol and collected by centrifugation. The OD of the cultures were monitored before the stress treatment and every 30 min for 2 h after stress treatment. Only samples from wild type and knock-out cultures which showed

similar growth rates ($\pm 10\%$) (Thompson et al., 2013) were used for transcriptome comparisons. Total RNA was extracted, quality controlled and quantified as described previously (Merhej et al., 2015). One microgram of total RNA was used for fluorescent cDNA synthesis according to the amino-allyl protocol (Merhej et al., 2015). The cDNA were labeled with Cy3 and Cy5 and hybridization was performed as previously described (Merhej et al., 2015). Two biologically independent experiments were performed for each condition, using dye switch. We used custom *C. glabrata* Agilent arrays in an 8 × 60 k format (array express accession number: A-MEXP-2402). After overnight hybridization and washing, the slides were scanned using a 2-micron Agilent microarray scanner. The images were analyzed using the feature extraction software (Agilent technologies) and normalized using global LOESS (Lemoine et al., 2006). The mean of the biological replicates was calculated. A gene was considered as being differentially expressed if its mean absolute Log₂(fold change) value was more than 0.75 and if its expression variation was considered as being statistically significant using the LIMMA package with a cut-off *p*-value of 0.02 (Ritchie et al., 2015). The complete microarray data are available at Array express database under the accession number: E-MTAB-4457.

TFBS Predictions

DNA sequences of ChIP peaks were retrieved from their genomic locations (BED file) using the “getfasta” function from the BEDTOOLS suite (Quinlan and Hall, 2010). These genomic sequences were used as inputs for the “peak-motif” tool to search for regulatory motifs (Thomas-Chollier et al., 2012). An additional filtering step was added to the standard peak motif procedure to discard low complexity motifs (e.g., CCCCCC) or motifs which were found in <20% of the peaks (Supplementary File S5).

Network Building

The ChIP peaks were assigned to genes as described previously (Merhej et al., 2014). When a peak was located in a divergent promoter (i.e., an intergenic region in between two divergent genes) the two genes were fused in one target in the network named “gene 1/gene 2,” unless we had transcriptome evidence supporting the regulation of one of the two genes. In this case, only the name of the regulated gene was kept. The network was represented using the igraph library (igraph.org, R programming language; Csardi and Nepusz, 2006), combining three different types of information (Supplementary Table 1). The ChIP parameter was used to define interactions (arrows) between the different Yap factors and their target promoters. The transcriptome parameter was used to color arrows depending on the directionality of the regulation (activation, repression, or no detected change). The TFBS parameter was used to color target promoters depending of the presence of the identified TFBS in the corresponding ChIP peaks.

Gene Ontology and Gene Set Enrichment Analyses

GO analyses were performed using the “GO term finder” tool at the CGD database, with default parameters (Inglis et al., 2012).

GSEA were performed using the GSEA module of the Jexpress software suite with a cut-off FDR of 1% (Subramanian et al., 2005; Stavrum et al., 2008). We used as input files transcriptome analyses of the responses of *C. glabrata* wild type cells to fluconazole (Kuo et al., 2010b), sodium salt (Roetzer et al., 2008; Wapinski et al., 2010), heat shock (Roetzer et al., 2008; Wapinski et al., 2010), hydrogen peroxide (Wapinski et al., 2010; Roetzer et al., 2011), menadione (Roetzer et al., 2011), glucose starvation (Roetzer et al., 2008), sorbic acid (Jandric et al., 2013), iron excess and iron starvation (this work, E-MTAB-4457), cadmium chloride and sodium selenite (Thiebaut et al., unpublished data).

RESULTS

The Yap Network in *C. glabrata*

To identify the gene targets and characterize the regulatory interactions of the seven Yap transcription factors of *C. glabrata*, we used three different approaches. First, we performed ChIP-seq experiments using myc-tagged versions of each factor. For CgYap1, CgYap2, CgYap4/6, and CgYap5, we submitted the corresponding tagged strains to stress conditions known to induce full activity of their *S. cerevisiae* orthologues, i.e., oxidative stress caused by a metalloid (namely selenium) for Yap1 (Haugen et al., 2004; Salin et al., 2008), cadmium for Yap2 (Azevedo et al., 2007; Mazzola et al., 2015), salt excess for Yap4/6 (Nevitt et al., 2004; Ni et al., 2009), and iron excess for Yap5 (Li et al., 2008; Pimentel et al., 2012). For CgYap7, which was shown to have a constitutive activity (Merhej et al., 2015), the experiments were performed in standard growth conditions. For the two orthologs of Yap3, whose role remains unknown in *S. cerevisiae*, the experiments were performed using cells grown in YPD and cells exposed to a pleiotropic stress inducer (selenite; Salin et al., 2008). The ChIP-seq data were analyzed taking as a reference both the input control and the mock IP control, to sort out a maximum of the false positive peaks due to highly expressed loci (Park et al., 2013; Teytelman et al., 2013). Second, we used the ChIP-seq data to predict the preferred Transcription Factor DNA binding sites (TFBS) for each CgYap. Reciprocally, we identified all the ChIP-peaks which contain the predicted consensus in their promoter sequence. Third, we compared the transcriptome of wild type and null mutants for each CgYap, using the same growth conditions as those used for ChIP-seq experiments. Hence, we identified the genes for which expression was altered, directly or indirectly, in the absence of the corresponding transcription factor.

To build a network from these three sources of information, we used a scoring system based on simple but meaningful logical rules (Supplementary Table 1). Briefly, a regulatory interaction was included in the network if it was detected by ChIP-seq. Then, the interactions and the edges were differently labeled depending on the transcriptome and TFBS data (Figure 1). As a consequence, the different interactions in the final network do not have the same value, depending on whether they were supported by one, two, or three experimental evidences (Supplementary Table 1). The final CgYap network contained 6 transcription factors and 255 regulatory interactions involving 214 promoters and 309 potential target genes (Figure 1). We could not identify any target gene for CgYap3a, neither by

ChIP-seq or by transcriptome analyses. Notably, 62% of the interactions in the network were supported by at least two evidences (Figure 1). The majority of the ChIP targets that we identified had only one peak in their promoter. However, in few cases, several binding sites could be unambiguously detected for CgYap1 and CgYap7 (Supplementary File S6). The number of interactions for each CgYap was highly variable, from 3 for CgYap2, up to 118 for CgYap7 (Figure 1). Relatively few redundancies were observed between the different Yaps, only 38 genes (18% of the edges) are targeted by more than one CgYap.

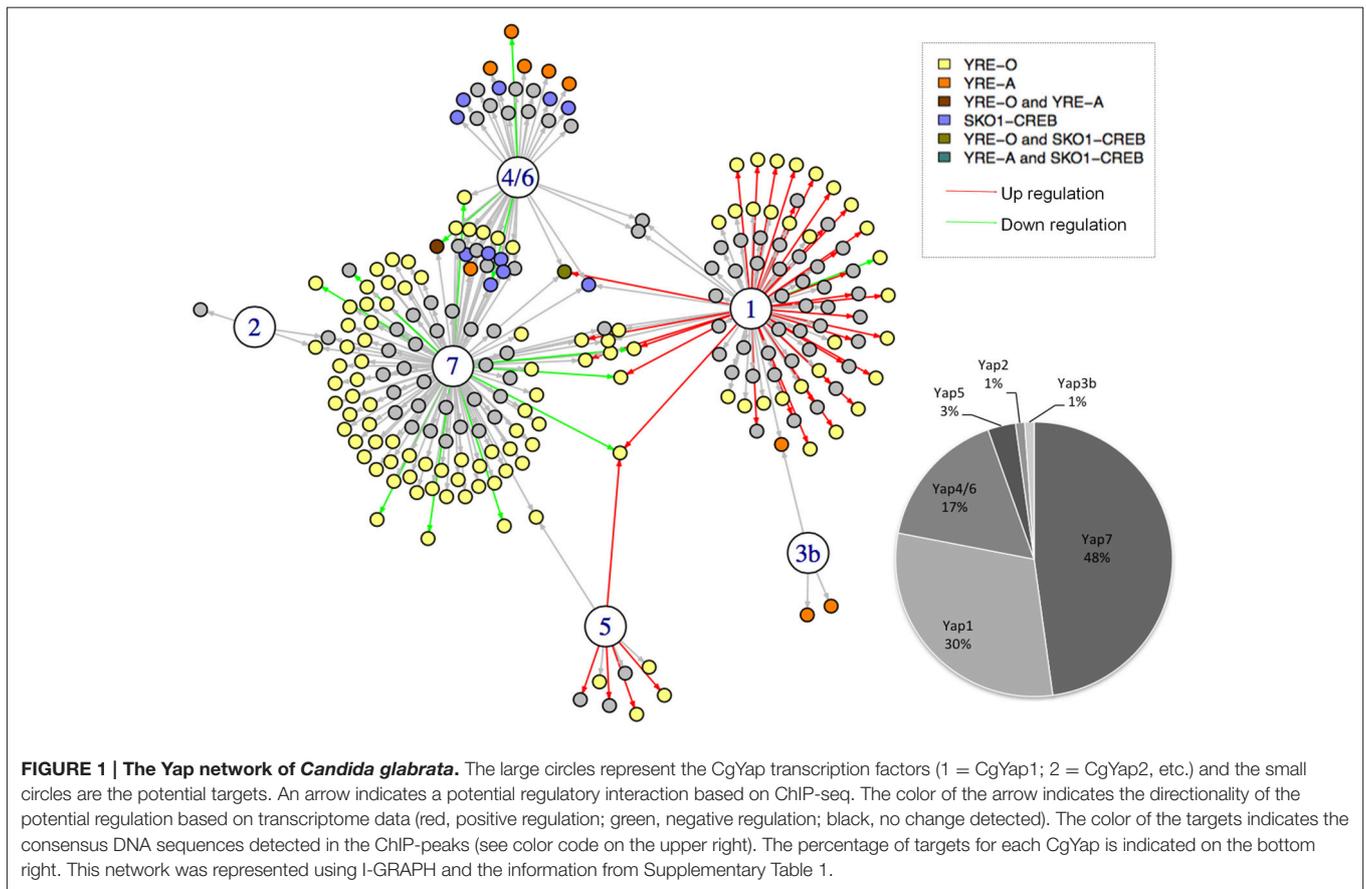
C. glabrata Yap Transcription Factor Binding Sites

As mentioned above, the peaks identified from ChIP-seq data were used to predict the TFBS for 5 of the 7 studied CgYap (Figure 2). In *S. cerevisiae*, the Yap proteins were classified in two categories based on their preferred TFBS (named YRE for Yap Response Elements): ScYap1, ScYap2, ScYap5, and ScYap7 bind to YRE-O (TTACTAA) motifs while ScYap3, ScYap4, and ScYap6 rather recognize YRE-A (TTACGTAA) motifs (Tan et al., 2008; Kuo et al., 2010a). We found that this dichotomy was remarkably conserved in *C. glabrata* (Figure 2). The predicted binding sites for CgYap1, CgYap5 and CgYap7 were very close to the perfect YRE-O consensus. In contrast, the YRE-A motif was enriched in the ChIP peaks of CgYap3b and CgYap4/6. These YRE motifs were identified as the best predicted motifs for all Yaps, except for CgYap4/6 (Figure 2, Supplementary File S5). For this transcription factor, the best identified motif was ATGACGTCAT, which differs from the canonical YRE-A motif by its higher GC content and which actually corresponds to the consensus motif published for another bZIP subfamily, the CREB/ATF2 factors (Fujii et al., 2000). Interestingly enough, in *S. cerevisiae* this motif was associated to the Sko1 transcription factor, which is a yeast homolog of the ATF2/CREB factors (Pascual-Ahuir et al., 2001; Gordan et al., 2011). Sko1 has been shown to contribute to the salt stress response of *S. cerevisiae*, together with ScYap4 and ScYap6, and to share a large number of targets with these factors (Ni et al., 2009).

The proportion of ChIP peaks containing the best predicted TFBS was remarkably high (from 41% for Yap1 up to 100% for Yap3b) (Figures 1, 2). This provided a posteriori confirmation that our ChIP-seq analyses procedure efficiently filtered out the false positive peaks.

Directionality of the CgYap Activities

Because we included transcriptome data in our network, we could determine the directionality (i.e., activation or repression) of some of the regulatory interactions set up by the different Yap proteins (Figure 1). This allowed us to predict the activator or repressor nature of these CgYaps. We observed that, in the conditions that were studied (selenite, excess of iron, salt stress, or YPD, respectively), CgYap1 and CgYap5 were strict activators, while CgYap4/6 and CgYap7 were strict repressors (Figure 1). The deletion of CgYap2 and CgYap3b had no effect on their targets in the conditions that we studied and therefore we had no information on their activity. The overlaps between transcriptome results and ChIP-seq results were remarkably



high in the sense that, taking into account the directionality mentioned above, between 40 and 90% of the expression changes observed in the transcriptome analyses involved genes to which promoters were bound by the TF according to ChIP-seq data (Supplementary File S7). The reciprocal was not true for CgYap7 and CgYap4/6, for which most of the ChIP targets showed unchanged expression in the mutant. This may be due either to functional redundancies for the regulation of these genes or because our transcriptome experiments were not conducted in conditions in which these regulations were active.

Functional Annotation of the Yap Network

We performed Gene Ontology (GO) term enrichment analyses on the whole set of genes in the network and on the individual sets of target of each CgYap (Figure 3, Supplementary Table 1). The whole network was enriched in GO categories related to oxido-reduction processes and iron homeostasis (Figure 3). CgYap1, CgYap5, and CgYap7 were the main contributors to these categories, while CgYap2, CgYap3b, and CgYap4/6 did not show any significant enrichment. More specifically, CgYap1 was the main contributor of targets related to oxidative stress response, oxido-reduction and chemical stress response (e.g., *TRR1*, *TRX2*, *OYE2*, *GPX2*, *TSA1*, *CTA1*, *SRX1*, ...). Its target set was also enriched in genes involved in heme biosynthesis (*HEM1*, *HEM3*, *HEM15*, and *HEM2*). CgYap5 target list was

clearly associated to iron sulfur cluster binding (*ISA1*, *TYW1*, *ACO1*, *RLI1*, *SDH2*, *GLT1*) and iron homeostasis (*GRX4*, *CCC1*). The only GO category to be enriched in CgYap7 targets was iron sulfur cluster metabolism. This includes genes involved in the cytosolic and mitochondrial iron sulfur assembly pathway (*CIA1*, *CIA2*, *DRE2*, *NAR1*, *CFD1*, *IBA57*, *JAC1*) and in iron sulfur cluster binding (*LYS4*, *SDH2*). CgYap7 also targets 10 genes encoding oxidoreductases (*CCP1*, *ERG11*, *YHB1*, *OYE2*, etc...) and genes encoding heme containing proteins (*YHB1*, *CCP1*) or related to heme metabolism (*HEM3*, *CYC3*). The targets of CgYap1 and CgYap7 included many genes encoding transcription factors. For instance, several transcription factors involved in oxygen homeostasis and oxidative stress responses are targeted by CgYap1 (*ROX1*, *MSN4*, *RPN4*, *SKN7*, *IXR1*). Remarkably, CgYap1 and CgYap7 both bound their own promoter, suggesting auto-regulation (Supplementary Table 1).

Conservation of the CgYap1/5/7 Subnetwork

The functional annotation presented above pointed out a CgYap1/CgYap5/CgYap7 network centered on iron and redox homeostasis. To assess the conservation of this sub-network we compared the targets of CgYap1, CgYap5, and CgYap7 with the targets of their orthologues in *S. cerevisiae* and *C. albicans* (Li et al., 2008, 2011; Salin et al., 2008; Znaidi et al., 2009; Chen

TF	Motifs in <i>C. glabrata</i> (this work)	Motif type	% of ChIP peaks with motif	Motifs in <i>C. glabrata</i> (previous studies)	Motifs in <i>S. cerevisiae</i> (previous studies)
Yap1		YRE-O	41%	YRE-A and/or YRE-O	YRE-O
Yap2	ND	NA	NA	ND	YRE-O
Yap3a	ND	NA	NA	ND	YRE-A
Yap3b		YRE-A	100%	ND	
Yap4/6		Sko1/CREB	46%	ND	YRE-A
		YRE-A	26%		
Yap5		YRE-O	67%	ND	YRE-O
Yap7		YRE-O	66%	ND	YRE-O

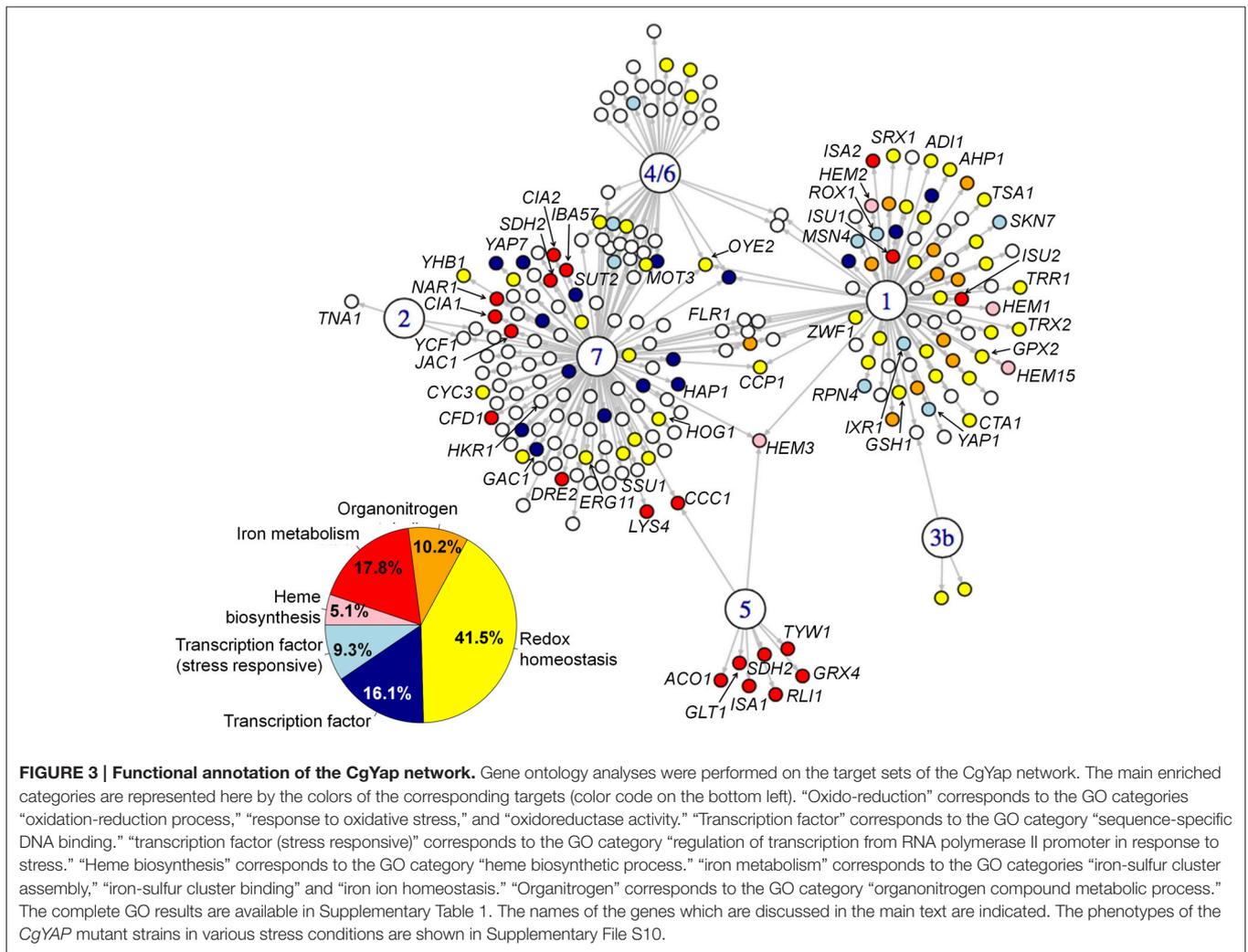
FIGURE 2 | CgYaps Transcription Factor Binding Sites predictions based on ChIP peaks. The motifs were predicted from the ChIP-peaks sequences using Peak Motif (Thomas-Chollier et al., 2012). The motifs previously found for the Yap transcription factors in *S. cerevisiae* and for CgYap1 in *C. glabrata* are indicated (Tan et al., 2008; Kuo et al., 2010a; Goudot et al., 2011). The complete Peak Motif predictions are available in Supplementary File S5.

et al., 2011; Hsu et al., 2011; Singh et al., 2011; Pimentel et al., 2012; Supplementary Table 2). The functional categories in which Yap1 and Yap5 are involved were remarkably conserved: ScYap1 and Cap1 are also involved in oxido-reduction processes (Salin et al., 2008; Znaidi et al., 2009), ScYap5 and Hap43 are involved in iron homeostasis, but ScYap5 is an activator of iron stress response while Hap43 has a role in repressing iron consuming genes in iron limiting conditions (Li et al., 2008; Hsu et al., 2011; Singh et al., 2011). No clear GO category could be attributed to ScYap7 besides its role in *YHB1* repression but all previous genome-wide studies (for instance Harbison et al., 2004) have been conducted in a strain background in which the *ScYAP7* gene is interrupted by a frame-shift mutation (Merhej et al., 2015). In terms of gene targets, the three Yap5 targets which had been validated in *S. cerevisiae* (*CCC1*, *GRX4*, and *TYW1*; Li et al., 2008, 2011; Pimentel et al., 2012) were conserved in *C. glabrata* (Figure 4A). Remarkably, despite the large evolutionary distance between *C. glabrata* and *C. albicans*, all CgYap5 targets, except *GRX4*, are targets of its orthologue Hap43 (Figure 4B). Hap43 also shares 19 targets with CgYap7, many of which are involved in iron sulfur cluster metabolism, redox homeostasis or heme metabolism (e.g., *NAR1*, *DRE2*, *LYS4*, *SDH2*, *SSU1*, *OYE2*, *YHB1*, *CCP1*, *HEM3*, ...).

Among the 82 targets that we identified for CgYap1, 28 are orthologous to targets of ScYap1 and 20 to Cap1 targets (Figures 4C,D). Of these, 15 were common to the three orthologs. This set of highly conserved Yap1 targets includes several enzymes known to play important roles in redox balance (*OYE2*, *TRX2*, *TRR1*, *CTA1*, *GPX2*, *GSH1*, *ZWF1*, *CCP1*), the MFS permease *FLR1* (named *MDR1* in *C. albicans*) and, remarkably, Yap1 itself, suggesting that the auto-regulation of Yap1 may actually play a significant role in its function. Besides this relatively high conservation of the Yap1 regulon in yeasts, a remarkable specificity of CgYap1 is its role in the direct regulation of several genes encoding enzymes of the heme biosynthetic pathway, a feature which was documented neither in *S. cerevisiae* nor in *C. albicans*.

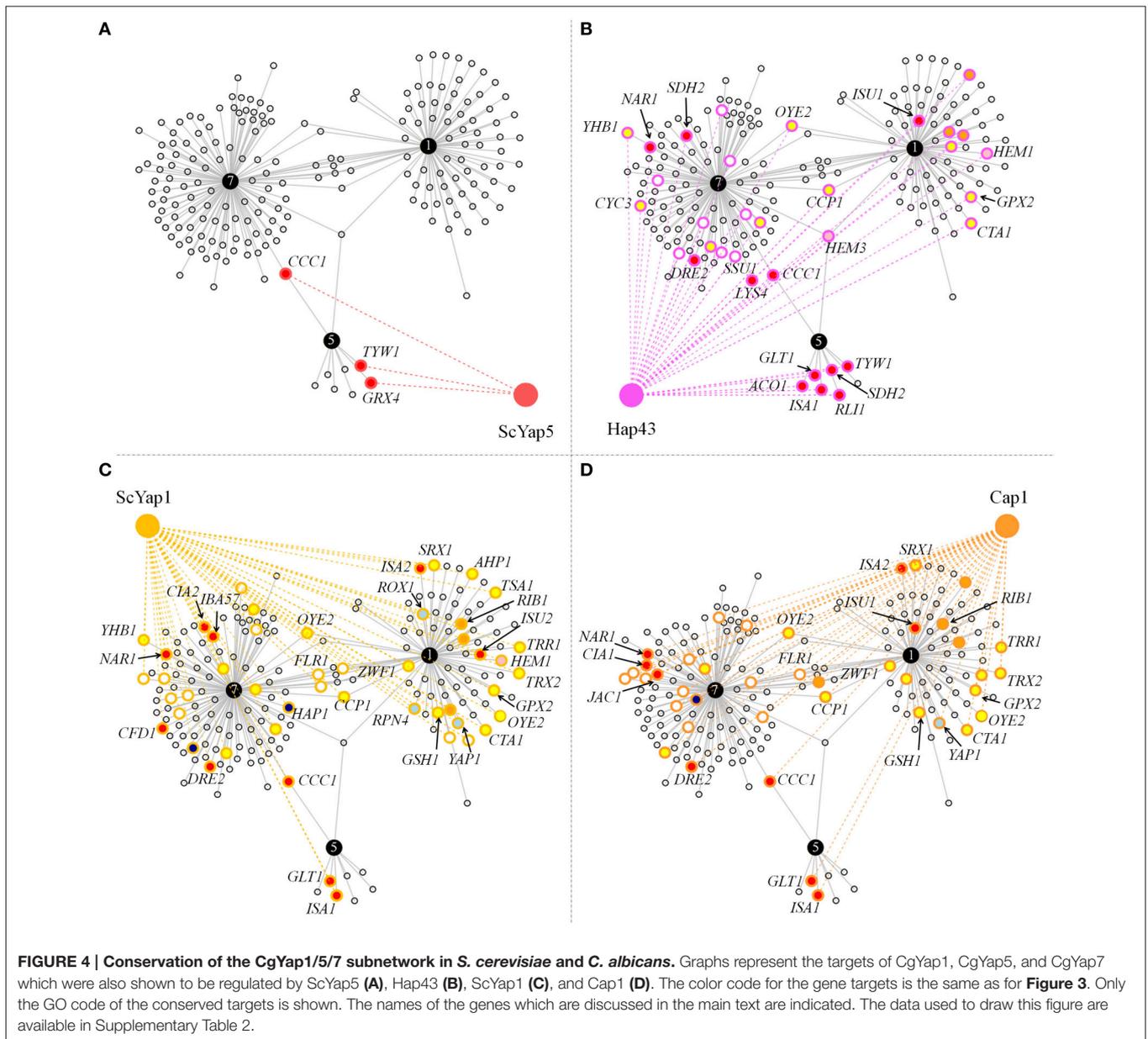
Overlap between the CgYap Network and Stress Responses in *C. glabrata*

We next used Gene Set Enrichment Analyses (GSEA; Subramanian et al., 2005) to look for enrichments of our CgYap target sets in transcriptome analyses of *C. glabrata* wild type cells to various environmental stresses. The list and origin of the transcriptome data which were used can be found in the methods. As could have been expected from the results presented



in the previous chapters, the CgYap1 targets were significantly enriched among the genes induced by oxidative stress causing agents (H₂O₂, selenite, iron, with the exception of menadione; Supplementary File S8), supporting the general role of this transcription factor in the oxidative stress response of *C. glabrata* (Lelandais et al., 2008; Roetzer et al., 2011). More surprisingly, the targets of CgYap1 were enriched in heat shock and sorbic acid stress responses. Interestingly enough, the HSE (Heat Shock Element) was found to be present in 25% of the CgYap1 ChIP peaks (Supplementary File S5). In *S. cerevisiae*, previous works have identified connections between Yap1 activity on one hand and Hsf1 and some gene regulatory modules induced by heat shock on the other (Hahn et al., 2006; Wu and Li, 2008; Nussbaum et al., 2014). Our results suggest that these connections might be conserved in *C. glabrata*. In contrast, the targets of CgYap4/6 were not particularly enriched in osmotic stress responses caused by NaCl excess (Supplementary File S8), which questions the conservation in *C. glabrata* of the role of the ScYap4 and ScYap6 in this process. As expected, the targets of CgYap5 were globally induced by iron excess in *C. glabrata* but we also observed that the CgYap5 set of targets

was significantly repressed in iron depleted conditions caused by BPS treatment or selenite exposure (Supplementary File S8, **Figure 5A**). Moreover, our transcriptome analyses of iron starvation and iron excess responses in wild type *C. glabrata* cells showed that CgYAP5 itself had expression levels which were inversely correlated to the iron concentration (**Figure 5B**), suggesting an active role for this transcription factor in iron starvation. Obviously, the fact that CgYap5 targets were repressed by iron starvation did not necessarily mean that CgYap5 was directly involved in this regulation. For instance, in *S. cerevisiae*, the repression of many iron consuming genes such as *ACO1*, *SDH2*, *ISA1*, and *CCC1* in iron starved conditions occurs post-transcriptionally and is mediated by the RNA binding proteins Cth1 and Cth2 (Puig et al., 2005). To assess the actual role of CgYap5 in the iron starvation response of *C. glabrata*, we analyzed the transcriptome response of Δ Cgyp5 cells to BPS treatment and compared this response with the one of wild type cells. We observed that most of the genes, which were dependent on Yap5 for their high iron induction, were similarly repressed by BPS in the wild type and in the Δ Cgyp5 mutant (exemplified by *SDH2* on **Figure 5C**), indicating that this

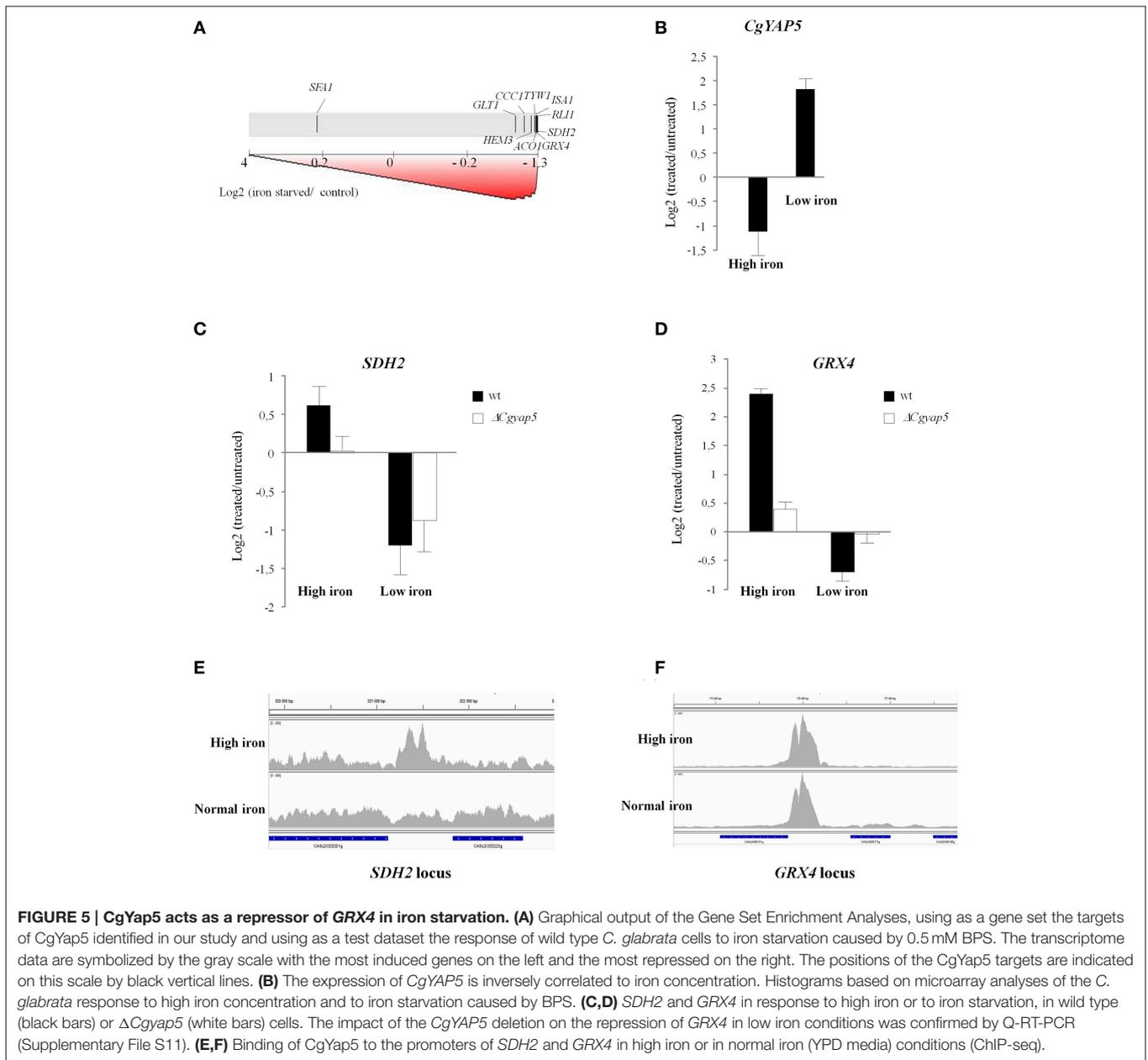


repression was not CgYap5 dependent. The only exception was *GRX4*, for which repression was totally abolished when CgYap5 was absent (Figure 5D). Consistent with these results, ChIP-seq experiments conducted in iron-replete conditions showed that the binding of CgYap5 to *GRX4* promoter was constitutive, while binding to its other targets was detected only at high iron concentrations (Figures 5E,F). Notably, the *GRX4* expression level was previously shown to be independent of CgYap5 in standard growth conditions (Merhej et al., 2015), indicating that the effect detected here was indeed specific of iron starvation. These results strongly suggest that, in addition to its role in the iron stress response, CgYap5 plays an active role in the iron starvation response by directly repressing the expression of *GRX4*.

DISCUSSION

A Methodology to Build Highly Consistent Regulatory Networks

Global ChIP and transcriptome analyses are powerful tools to achieve comprehensive descriptions of large transcriptional regulatory networks (Babu et al., 2004; Harbison et al., 2004). However, the interpretation of these networks is dampened by the tendency of these techniques to generate large numbers of false positives. For instance, highly expressed genomic regions (tRNA genes, glycolytic enzymes encoding genes, etc.) have been shown to be nonspecifically enriched in ChIP-seq experiments, leading to tens to hundreds of misidentified “targets” (Park et al., 2013; Teytelman et al., 2013). This bias is



better captured, but only partially corrected, using mock IP as reference for peak calling, rather than input DNA (Park et al., 2013; Krebs et al., 2014). For transcriptome analyses, relatively minor differences between wild type and mutant growth rates or stress response dynamics can eventually produce tens of differentially expressed genes which have no real relationship with the mutation being tested (Thompson et al., 2013). We took into account these previous observations and designed experimental and bioinformatics procedures in which, 1- peak calling was performed using both the input DNA and the mock IP as references to efficiently sort out peaks corresponding to tRNA or highly expressed ORFs loci; and 2- only wild type and mutant cell cultures having very similar growth rates before and after the stress treatment were compared in transcriptome

analyses. These simple precautions led to a final network showing unusually high consistency between ChIP results and TFBS predictions on the one hand, and ChIP and transcriptome results on the other hand. Indeed, between 40 and 100% of the ChIP peaks identified contained one or several YRE (Yap Response Element). For comparison, the rate of YRE containing peaks in previous ChIP-chip studies conducted on the Yaps of *S. cerevisiae* (Tan et al., 2008; Ni et al., 2009) or on Yap1 in *C. glabrata* (Kuo et al., 2010a) ranged from 15 to 30%. Similarly, between 40 and 90% of the genes for which the transcriptome data showed an expression change had a ChIP peak in their promoter. This percentage ranged from 0 to 25% in a previous study of the *S. cerevisiae* Yap family (Tan et al., 2008).

These data allowed us to propose targets with a reasonably high level of confidence for 6 of the 7 Yaps in *C. glabrata*, to predict the preferred binding motifs of 5 of them and identify enriched functional categories for CgYap1, CgYap5, and CgYap7.

Conserved DNA Binding Properties of the Yap Transcription Factors

TFBS predictions based on the ChIP peaks pointed out a perfect conservation in the DNA binding properties of Yap1, Yap3b, Yap4/6, Yap5, and Yap7. As in *S. cerevisiae* (Tan et al., 2008), CgYap1, CgYap5, and CgYap7 were predicted to recognize preferentially the YRE-O site (TTACTAA) while CgYap3b and CgYap4/6 rather binds YRE-A (TTACGTAA). This result is consistent with the high conservation of the DNA binding domains of these proteins between *C. glabrata* and *S. cerevisiae* (Kuo et al., 2010a). Yet, this was unexpected in the case of CgYap1. Indeed, it was previously proposed, based on bioinformatic predictions from ChIP-chip data, that CgYap1 shifted its binding preferences from YRE-O to YRE-A due to a single mutation in the DNA binding domain compared to ScYap1 (Kuo et al., 2010a). This model was toned down by further analyses of the same dataset, which showed that YRE-O were as frequent as YRE-A in the promoters of CgYap1 targets (Goudot et al., 2011). Our ChIP-seq data unambiguously suggest that YRE-O is the preferred DNA binding site of CgYap1 and that the co-evolution scenario previously published has to be reconsidered. The discrepancy between these different studies may rely on the size of the genomic sequences which were used for TFBS predictions. Indeed previous ChIP-chip data provided peaks which were as wide as intergenic regions (800 base pairs in Goudot et al., 2011) while the binding regions identified by our ChIP-seq analyses for CgYap1 were 300 base pairs in average, leading to a much more precise identification of the actual binding location of the transcription factor (Supplementary File S4).

The Yap1 Core Regulon

Hence, the DNA binding properties of Yap1 were remarkably conserved between *S. cerevisiae*, *C. glabrata*, and *C. albicans*, since the YRE-O was also shown to be the preferred binding motif of Cap1 (Znaidi et al., 2009; Goudot et al., 2011). What was true for DNA binding was also true at the level of gene targets and functional annotations. Indeed, our data confirmed the role of CgYap1 in redox homeostasis, as previously demonstrated (Chen et al., 2007; Lelandais et al., 2008; Kuo et al., 2010a; Roetzer et al., 2011). We identified a core Yap1 regulon of 28 targets between *S. cerevisiae* and *C. glabrata* and 15 conserved targets between *S. cerevisiae*, *C. glabrata*, and *C. albicans*. Notably, this enlarged by more than two-fold the list of conserved Yap1 targets which were previously identified (Kuo et al., 2010a; Goudot et al., 2011). This core Yap1 regulon is composed mostly of well-known and highly conserved actors of oxidative stress response such as glutathion peroxidase and glutathion synthetase, catalase, mitochondrial peroxydase, enzymes of the thioredoxin pathway (thioredoxins, thioredoxin reductase, thioredoxin peroxydase, and thioredoxin peroxydase reductase), the FLR1/MDR1 permease, enzymes involved in NADPH metabolism (NADPH oxydoreductase of the Old Yellow

Enzyme family and glucose phosphate dehydrogenase). This confirms that, as suggested by previous work, oxidative stress response in general and the Yap1 control of this response in particular, do not fundamentally differ in *C. glabrata* compared with *S. cerevisiae* and *C. albicans* (Cuellar-Cruz et al., 2008; Lelandais et al., 2008; Kuo et al., 2010a; Gulshan et al., 2011; Roetzer et al., 2011; Briones-Martin-Del-Campo et al., 2014). Also, Yap1 binding to its own promoter, which had been demonstrated in *C. albicans* and *S. cerevisiae* (Salin et al., 2008; Znaidi et al., 2009), was conserved in *C. glabrata*. Although the primary activation of Yap1 has been shown to be at the post-translational level (Kuge et al., 1997; Zhang et al., 2000), this high conservation suggests that Yap1 autoregulation could play a role in conditions of acute oxidative stress, such as the ones used in the aforementioned studies. In support to this hypothesis, ScYAP1, CgYAP1 and CAP1 were all shown to be induced by oxidative stress at the mRNA level (Salin et al., 2008; Znaidi et al., 2009).

Insights in the Roles of Yap2 and Yap4/6 in *C. glabrata*

Only three ChIP targets could be detected for CgYap2 and CgYap3b, which did not allow us to propose functional annotations for these two factors. Still, it is interesting to notice that CgYap2 targets *YCF1*, which encodes a vacuolar transporter playing a key role in cadmium detoxification in *S. cerevisiae* (Wemmie et al., 1994; Li et al., 1997) and *TNA1*, the inactivation of which leads to cadmium sensitivity (Ruotolo et al., 2008). This suggests that the role of ScYap2 in cadmium resistance is conserved in *C. glabrata*. In *S. cerevisiae*, the activity of Yap2 is hidden by its partial functional redundancy with Yap1 (Azevedo et al., 2007; Iwai et al., 2010; Mazzola et al., 2015). Hence, it would be interesting to conduct Δ Cgyap2 transcriptome analyses in a context in which CgYAP1 has been knocked out.

We identified about 40 potential targets for CgYap4/6. The enrichment of the Sko1 binding motifs in the ChIP peaks indicated that the role of ScYap4 and ScYap6 in osmotic stress response may be conserved in *C. glabrata*. However, our GSEA and GO analyses showed that the CgYap4/6 targets were enriched neither in NaCl responsive genes nor in any particular functional category and the actual role of this factor remains to be elucidated. Our transcriptome analyses suggested that CgYap4/6 acts as a transcriptional repressor in the conditions that we tested. In *S. cerevisiae*, this point is controversial. ScYap4 and ScYap6 have been shown to recruit the general transcriptional repressor Tup1 (Hanlon et al., 2011) and bioinformatic analyses based on transcriptome data have suggested that they could be both repressors and activators (Tan et al., 2008). However, previous studies based on northern blots had shown that ScYap4 positively impacts the expression of three genes in response to osmotic shock (Nevitt et al., 2004).

CgYap5 Can Act Both as an Activator and a Repressor of Glutaredoxin Expression, Depending on Iron Availability

Similarly to CgYap1, CgYap5 has a conserved role in the iron stress response. In *S. cerevisiae*, Yap5 acts at three levels against iron excess by 1- the induction of the glutaredoxin Grx4

which senses iron-sulfur clusters in the cytoplasm (Muhlenhoff et al., 2010) and negatively controls the activity of the Aft1/2 transcriptional activators of iron uptake (Ojeda et al., 2006; Ueta et al., 2012); 2- the induction of Ccc1 which transports cytoplasmic iron into the vacuole (Li et al., 2008); and 3- the overexpression of Tyw1 which is an iron-sulfur cluster containing protein and therefore contribute to iron sequestration (Li et al., 2011). We showed here that, in iron excess conditions, CgYap5 similarly controls the expression of *GRX4*, *CCC1*, and genes encoding proteins involved in iron sequestration either through iron-sulfur cluster binding and biogenesis (*TYW1*, *GLT1*, *ACO1*, *RLI1*, *SDH2*, *ISA1*) or through heme biosynthesis (*HEM3*).

In addition to this conserved role in the detoxification of iron excess, we showed that CgYap5 is overexpressed in response to iron starvation and that it represses the expression of *GRX4* in these conditions. As mentioned above, Grx4 inhibits the iron starvation response by promoting the nuclear export of Aft1/2 when iron-sulfur clusters are abundant (reviewed in Lill et al., 2014). The regulation of glutaredoxin activity is mostly post-translational (Lill et al., 2014), but transcriptional repression of *GRX4* by CgYap5 may provide a supplementary layer of regulation to ensure full Aft1/2 activity in iron starvation conditions (Figure 6). This new role of Yap5 may be conserved in *S. cerevisiae*, since ScYap5 has been shown to bind the *GRX4* promoter independently of iron concentration and since the deletion of ScYAP5 negatively impacts the nuclear localization of Aft1/2 in iron limiting conditions (Pimentel et al., 2012). However, the inactivation of ScYAP5 does not seem to impact *GRX4* expression in iron-replete cells (Pimentel et al., 2012).

This dual role of CgYap5 is reminiscent of its HapX orthologs. HapX was initially identified as a key regulator of iron starvation response in filamentous ascomycetes (Hortschansky et al., 2007; Schrettl et al., 2010; Lopez-Berges et al., 2012), basidiomycetes (Jung et al., 2010), and hemiascomycetes of the *C. albicans* clade (where it is called Hap43; Hsu et al., 2011; Singh et al., 2011). HapX acts by repressing iron consuming genes when iron is limiting and HapX proteins are more expressed in iron starvation than in iron excess growth conditions (Singh et al.,

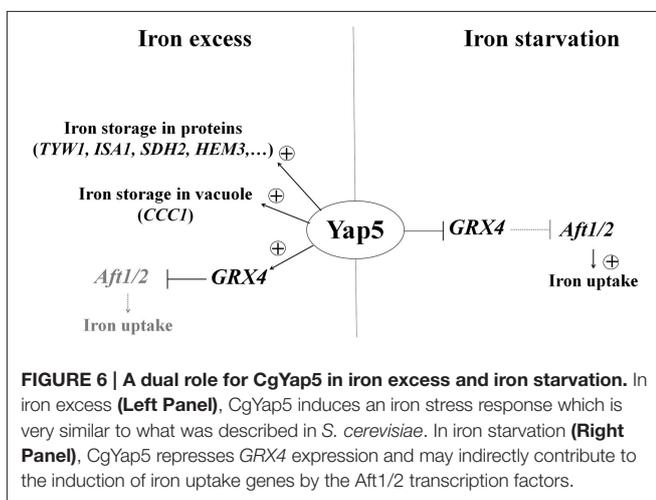
2011; Gsaller et al., 2014). However, HapX was shown recently to play an additional role in activating the expression of some of its targets in response to iron excess in *Aspergillus* and *Fusarium* sp. (Gsaller et al., 2014). Interestingly, Yap5 and HapX both sense iron by directly binding iron-sulfur clusters through a conserved cysteine rich domain (CRD; Gsaller et al., 2014; Rietzschel et al., 2015). Our data indicate that Yap5 and HapX may have more in common than just this CRD. As indicated above, CgYap5 is also overexpressed and able to repress transcription in iron starved cells (Figure 6). Moreover, all the CgYap5 targets that we identified, except *GRX4*, are targets of Hap43. This conservation of targets is remarkable considering that Hap43 and Yap5 have different DNA binding properties: Hap43, like HapX in other fungi, mainly binds CCAAT boxes through its Hap4L like domain (Hortschansky et al., 2007, 2015; Chen et al., 2011), while Yap5 binds YRE-O using exclusively its bZIP region (this work, Li et al., 2008; Pimentel et al., 2012). Still, the role of CgYap5 in iron starvation is limited to a modest repression of *GRX4* expression, while HapX strongly represses the expression of tens of iron consuming genes in these conditions (Supplementary File S9).

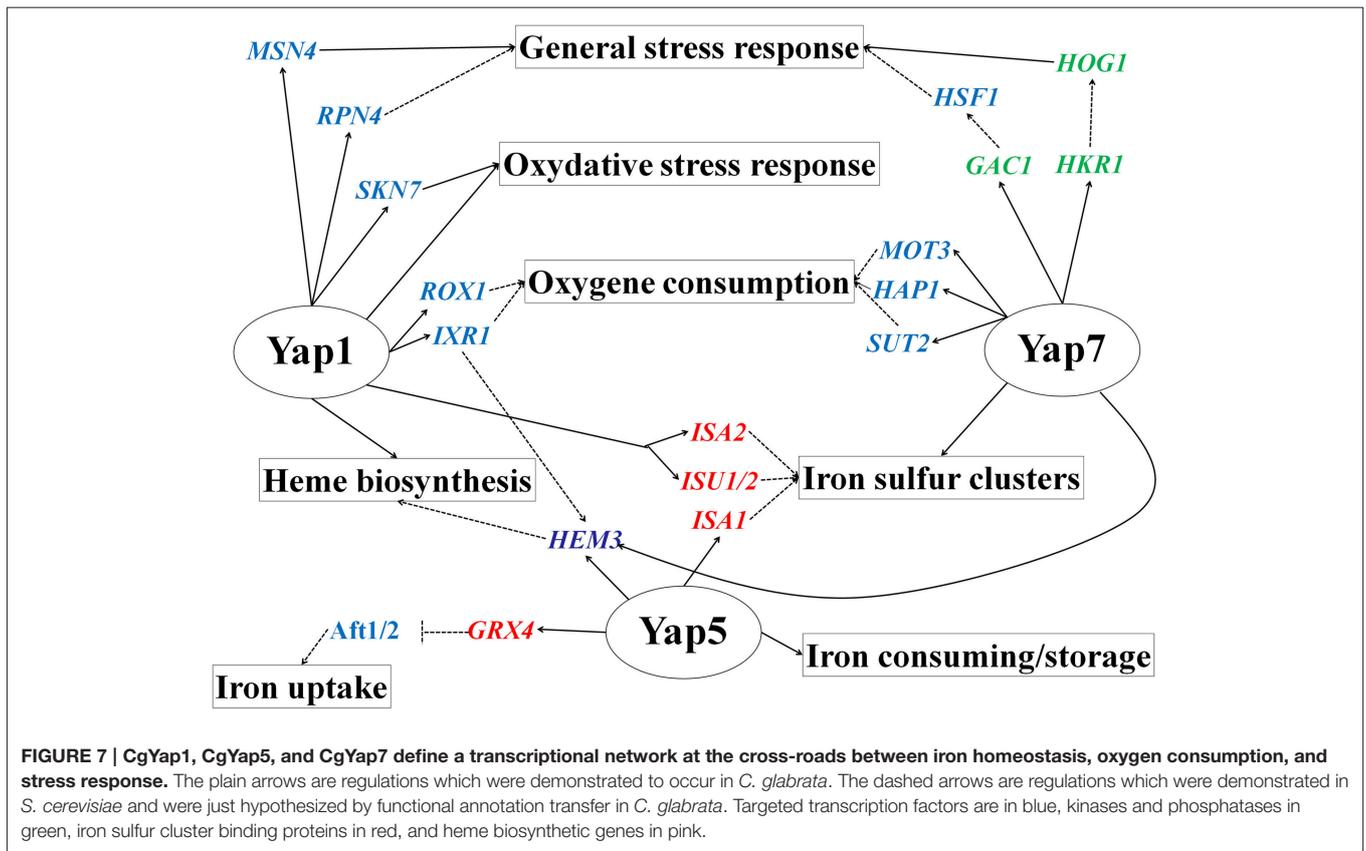
This new role of Yap5 opens the question of the molecular mechanisms which would allow CgYap5 to be a transcriptional repressor when iron is limiting. Several hypotheses can be mentioned, based on the literature. The transcriptional repression by Hap43 requires its Hap4L domain and involves the CCAAT binding complex (Singh et al., 2011). Yap5 only has a vestigial Hap4L domain (Merhej et al., 2015), which was supposed to be non-functional although its activity has actually never been tested so far. Second, Yap7, the ohnolog of Yap5, has been shown to repress transcription by recruiting the general repressor Tup1 (Merhej et al., 2015). Moreover, functional connections between Hap43 and Tup1 have been reported in *C. albicans* (Hsu et al., 2011). Additionally, the shift of Yap5 from an activator to a repressor could be controlled by iron availability, since the binding of iron-sulfur clusters to ScYap5 was shown to significantly change its conformation (Rietzschel et al., 2015).

Interconnections between CgYap1, CgYap5, CgYap7, and the *C. glabrata* Stress Response Network

Our data led us to propose new roles for Yap1 and Yap7 in the regulation of genes involved, respectively, in heme biosynthesis and in the biogenesis of iron-sulfur cluster proteins. Previously, Yap7 was shown to be a repressor of the heme-containing nitric oxide oxidase Yhb1 in *C. glabrata* and *S. cerevisiae* (Merhej et al., 2015). Our ChIP-seq data indicated that CgYap7 binds many genes encoding enzymes of the cytoplasmic and mitochondrial iron sulfur cluster biogenesis pathway. This result is particularly interesting considering the important role played by the mitochondrial iron sulfur clusters in the sensing of iron availability and in regulating the activity of Yap5, the ohnolog of Yap7 (Lill et al., 2014; Rietzschel et al., 2015).

In *S. cerevisiae*, there is little evidence of a connection between Yap1 and heme biosynthesis, besides the binding of ScYap1 to *HEM1* (Salin et al., 2008) and the regulation by





ScYap1 of the heme-dependent repressors *IXR1* and *ROX1* (Castro-Prego et al., 2010; Caetano et al., 2015). We show here that CgYap1 directly targets four of the eight enzymes involved in this pathway (*HEM1*, *HEM2*, *HEM3*, *HEM15*) and CgYAP1 deletion clearly impacted the expression of three of them (*HEM1*, *HEM3*, and *HEM15*). Hence, we defined here a CgYap1/CgYap5/CgYap7 network deeply involved in oxygen and iron sensing by tuning redox homeostasis, heme biosynthesis, iron storage, and iron sulfur cluster metabolism (Figure 7). Each factor has its own specificity (CgYap1 in redox balance and heme biosynthesis, CgYap5 in iron storage and CgYap7 in iron-sulfur clusters biogenesis) but there are some interconnections between them. For instance, CgYap5 and CgYap1 positively control the mitochondrial iron-sulfur cluster proteins maturation factors *Isa1* and *Isu1/2/Isa2*, respectively. Also, the *HEM3* gene, encoding the prophobilinogen deaminase (third step of heme biosynthesis) came out as a “hub” in this network, being positively regulated by CgYap1 and CgYap5 in response to oxidative stress or iron excess, respectively, and repressed by CgYap7 in standard growth conditions (Figure 3). Similarly, *CCP1*, encoding the cytochrome c peroxydase which acts as a heme-based sensor of the mitochondrial oxidative stress (Martins et al., 2013), is positively regulated by CgYap1 and repressed by CgYap7 (Figure 3).

Another interesting feature of CgYap1 and CgYap7 is the strong enrichment for transcription factors among their targets. For instance, they potentially control the expression of several

transcription factors known in *S. cerevisiae* for being involved in the regulation of hypoxic genes and oxygen consumption (*Rox1* and *Ixr1* for CgYap1 and *Mot3*, *Hap1* and *Sut2* for CgYap7; Figure 7; Castro-Prego et al., 2010; Gonzalez Siso et al., 2012). They also target some regulators involved in the general Environmental Stress response. This is for instance the case of the *Rpn4* and *Msn4* transcription factors for CgYap1 or of the *Hog1*, *Hkr1*, and *Gac1* stress signaling proteins for CgYap7. Additionally, CgYap1 seems to positively control the expression of *SKN7*, which encodes a regulator of oxidative stress response known to cooperate with Yap1 both in *S. cerevisiae* and in *C. glabrata* (Morgan et al., 1997; Lee et al., 1999; Saijo et al., 2010; Roetzer et al., 2011). This suggests that the network described here is tightly connected to other transcriptional responses and that CgYap1 and CgYap7 are master regulators in the *C. glabrata* hierarchy of transcription factors (Jothi et al., 2009; Bhardwaj et al., 2010).

AUTHOR CONTRIBUTIONS

JM constructed the strains and performed the ChIP-seq and transcriptome analyses. AT performed transcriptome analyses. CB, JB, MA, and SL performed the high-throughput sequencing. GL and JC performed the bioinformatics analyses and the network building. FD designed the experiments and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2016.00645>

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